REMARKS

Applicants thank Examiner Pak for the courtesy of a telephonic interview with Applicants' attorneys, Diana Collazo and Janis Fraser, on April 20, 2006, during which the outstanding rejections under 35 U.S.C. §112, first and second paragraphs, were discussed. Although an agreement with respect to the claims was not reached during the interview, Applicants thank the Examiner for her suggestions and valuable input on how to respond to the outstanding rejections.

Claims 15, 28, 30, and 37-38 have been amended. New claims 39-47 have been added. Upon entry of this amendment, claims 15-16, 18, 21, and 25-47 will be pending. No new matter has been added.

Support for amended claims 15, 30 and 37-38 can be found, for example, starting at page 14, second full paragraph, through page 15, lines 1-5, of the specification. Support for claim 28, as amended, can be found, e.g., starting at page 7, line 28 through page 8, line 2; page 13,
lines 13-24; and page 23, lines 6-10, of the specification. Support for new claims 39-47 can be found, e.g., at page 4, lines 15-27; page 12, lines 13-28, and Example 1 (page 30, line 26, to page 31, line 13) of the specification.

The claim amendments and cancellations made herein have been made solely to expedite prosecution of the instant application and should not be construed as an acquiescence to any of the Examiner's rejections.

Claim Rejections under 35 U.S.C. §112, Second Paragraph

On pages 2-4 of the outstanding Office Action, the Office has rejected claims 15, 30 and 37-38 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the invention. Each ground for this rejection is addressed individually below.

a. Claims 15 and 30 (and claims depending therefrom) were rejected because the metes and bounds of the phrase "hybridizes under stringent conditions" are allegedly unclear. This rejection has been met by amending claims 15 and 30 to specify the stringency conditions, as discussed with the Examiner during the telephonic interview. Therefore, all of

the claims reciting hybridization now specify the stringency conditions under which the polynucleotide encoding the (R)-2-octanol dehydrogenase used in the claimed methods hybridizes to the probe, thereby rendering this aspect of the rejection moot. The Examiner agreed that this language would take care of the indefiniteness issue.

b. Claims 15 and 37-38 were rejected as being allegedly unclear in their recitation of the phrase "polypeptide comprising the amino acid sequence of SEQ ID NO:2 with up to 50/10 conservative amino acid substitutions." According to the Examiner, it is not clear:

> [I]f the "polypeptide" is a variant of SEQ ID NO:2 or if the "polypeptide" has the amino acid sequence of SEQ ID NO:2. If the latter is true, since the polypeptide must have the amino acid sequence of SEQ ID NO:2, said polypeptide can not have any amino acid substitutions. (Office Action at page 3)

In response, claims 15 and 37-38 (and claims depending therefrom) have been amended to specify that the (R)-2-octanol dehydrogenase used in the claims methods comprises "an amino acid sequence that is a variant of SEQ ID NO:2" with the specified number of conservative amino acid substitutions. The Examiner agreed that this language would take care of the indefiniteness issue. Therefore, this second aspect of this rejection has been met.

c-d. Claim 28 was rejected because the phrases "substantially pure" and "chemically treated" allegedly render the claim indefinite. Claim 28 has been amended to read "at least 75% pure" and to replace the phrase "chemically treated" with "treated with an organic solvent," as discussed with the Examiner. Thus, these aspects of the rejection have been obviated.

In view of the claim amendments set forth above, Applicants respectfully request that the rejection of the claims under 35 U.S.C. §112, second paragraph, be withdrawn.

Rejection of Claims 15-16, 18, 21, 25-34 and 37 under 35 U.S.C. §112, First Paragraph

The Office has rejected claims 15-16, 18, 21, 25-34 and 37 under 35 U.S.C. §112, first paragraph, for alleged lack of enablement. According to the Examiner:

> [T]he specification, while being enabling for a method for the producing a (S)-4-halo-3-hydroxybutyric acid ester derivative using a (R)-2-octanol dehydrogenase having the amino acid sequence SEQ ID NO:2 or a transformant producing said enzyme, does not

> reasonably provide enablement for a method for the production of (S)-4-halo-3-hydroxybutyric acid ester derivative using a (R)-2-octanol dehydrogenase having 70-95% sequence identity to SEQ ID NO:2 or a (R)-2-octanol dehydrogenase encoded by a polynucleotide that hybridizes to SEQ ID NO:2 under any hybridization conditions or a transformant producing said enzyme. (Office Action at pages 4-5).

The Examiner indicated at pages 4-5 of the Office Action that the specification adequately enables claims directed to methods of producing an (S)-4-halo-3-hydroxybutyric acid ester derivative using an (R)-2-octanol dehydrogenase having the amino acid sequence of SEQ ID NO:2 (in this case, claims 35-36). The Examiner, however, contends that the claims directed to methods of using a dehydrogenase having an amino acid identity of at least 70-95% to SEQ ID NO:2 are not enabled. Implicit in the Examiner's statement is that the specification enables those method claims that require the dehydrogenase sequence to have an amino acid identity higher than 95%. Accordingly, claim 38, which is directed to a method of producing an (S)-4-halo-3-hydroxybutyric acid ester derivative using an (R)-2-octanol dehydrogenase that is a variant of SEQ ID NO:2 with up to 10 conservative amino acid substitutions (corresponding to about 96% sequence identity to SEQ ID NO:2, with all substitutions being conservative ones) should not raise any enablement issues. Confirmation of Applicants' understanding is respectfully requested.

With respect to the remaining claims, the Examiner states that:

> The claims encompass a method for producing (S)-4-halo-3-hydroxybutyric acid ester derivatives using any and all recombinants, variants and mutants of SEQ ID NO:2 having up to 30% amino acid modifications of SEQ ID NO:2 or any or all recombinants, variants and mutants of SEQ ID NO:2 encoded by a polynucleotide that hybridizes to SEQ ID NO:2 under any hybridization conditions.

Applicants respectfully traverse this aspect of the rejection. The rejected claims, as amended, are directed to methods of producing (S)-4-halo-3-hydroxybutyric acid ester derivatives using an (R)-2-octanol dehydrogenase that has the molecular weight specified and meets at least one of the following criteria: (i) is encoded by a polynucleotide that hybridizes under the stringency conditions specified to a nucleic acid probe consisting of the complement of the nucleotide sequence of SEQ ID NO:1 (claims 15 and 30); (ii) includes an amino acid sequence at least 70%-95% identical to the amino acid sequence of SEQ ID NO:2 (claims 15 and

31-34); or (iii) includes an amino acid sequence that is a variant of SEQ ID NO:2 with up to 30 or 50 conservative amino acid substitutions (claims 15, 37 and 39). It is noted that claims 15 and 30 have been amended to recite specific hybridization conditions; thus, the Examiner's rejection of these claims as encompassing "any or all recombinants, variants and mutants of SEQ ID NO:2 encoded by a polynucleotide that hybridizes to SEQ ID NO:2 under any hybridization conditions" no longer applies. Applicants submit that the claims, as amended herein, are commensurate in scope with the enablement provided in the specification for at least the reasons discussed during the interview and presented in more detail below.

The specification discloses the amino acid and nucleotide sequence of an (R)-2-octanol dehydrogenase and its extensive characterization as an enzyme that can reduce 4-haloacetoacetic acid esters to produce (S)-4-halo-3-hydroxybutyric acid ester derivatives having high optical purities. Techniques for manipulating the sequences disclosed are routine in the molecular biology field and are disclosed in the specification, for example, at page 14, lines 3-11. Assays for measuring enzymatic reduction activity are disclosed in the specification, for example, at page 11, lines 24-30. As discussed in more detail below, alcohol dehydrogenases are a class of enzymes that exhibit similar biological activity among members of this class, even if their overall sequence identity to each other is relatively low. Therefore, generating mutant alcohol dehydrogenases that retain the desired activity and are within the scope of the claims is a simple matter of manipulating the sequences provided in the specification using standard molecular biology techniques, and testing such mutants using the assays disclosed in the specification. Such level of routine experimentation can be practiced by the skilled artisan, without undue experimentation.

The specification further discloses that the (R)-2-octanol dehydrogenase amino acid sequence of SEQ ID NO:2 shows closest homology to the glucose dehydrogenase sequence of *Bacillus subtilis* (i.e., showing 43% identity and 61% homology) (specification at page 16, lines 1-5). An alignment of the amino acid sequences for SEQ ID NO:2 and the glucose dehydrogenase is submitted herewith as Appendix A. Based on sequence homology, molecular weight and other biochemical properties, the skilled artisan would have recognized at the filing date of the instant application that the (R)-2-octanol dehydrogenase used in the claimed methods was a member of the short-chain alcohol dehydrogenase class (page 155 from Hummel, W.

(1997) *Advances in Biochemical Engineering/Biotechnology* Vol. 58:147-184, submitted herewith as Appendix B). This reference explains at pages 155-156 that, although members of the short-chain alcohol dehydrogenase family share low overall amino acid sequence identity, they generally exhibit similar biological activities, as long as certain "core" regions are conserved. These "core" regions were known at the priority date to be located at, for example, the N-terminal region of the protein, both in terms of secondary structure and linear amino acid sequence. For example, the Hummel review states that:

> However related with respect to their subunit size and coenzyme binding motif, all these enzymes are very dissimilar regarding the residue identities of 15-35% only....The secondary structure of the Drosophila ADH was predicted by Thatcher and Sawyer [110] who suggested an alternating βαβ region (a nucleotide-binding domain) at the N-terminus of the protein. This assumed function of the N-terminal part of these enzymes was supported by a conserved glycine-rich domain (GXXXGXG) found in most of the short chain dehydrogenases.
>
> The recent elucidation of a large number of short-chain ADH sequences permits a detailed characterization of this enzyme group. As many as six domains are found to be significantly conserved. A domain located at the N-terminus with a length of approximately 30 amino acids is generally assumed to be the coenzyme binding site. A second conserved domain is a hydrophobic region comprising 10 or 11 residues, respectively. A further conserved domain consists of 18 amino acids are probably involved in the dimerization of enzyme subunits or facilitate the hydride transfer. It starts with a totally conserved tyrosine and ends with a highly conserved acidic amino acid. (pages 156-57 of Hummel, W. (1997) *supra*)

The conserved glycine-rich domains at the N-termini of SEQ ID NO:2 and the glucose dehydrogenase of *Bacillus subtilis* are indicated by the boxed annotation in Appendix A. Other highly conserved amino acid regions are indicated throughout the alignment. Therefore, at the time the present application was filed, one of ordinary skill in the art would have known to make changes preferentially outside of the highly conserved amino acid stretches of SEQ ID NO:2, thus generating mutants that retain the desired dehydrogenase activity and that have an amino acid sequence at least 70% identical to SEQ ID NO:2, or that is encoded by a sequence that hybridizes to the complement of SEQ ID NO:1 under the stringency conditions specified. Such mutants could be tested using the assays described and exemplified in the instant specification.

To further illustrate the high degree of variability in amino acid sequence tolerated by alcohol dehydrogenases, while retaining similar biological activities, a paper by Cho, J. *et al.* (1998) *Applied and Environmental Microbiology* Vol. 64, No. 4:1350-1358 is submitted herewith as Appendix C. This paper shows that two alcohol dehydrogenase isozymes from *Pichia stipitis* appear to be equivalent in their ability to convert ethanol to acetaldehyde, even though their amino acid sequences are only 80.5% identical to one another. *Id.* at page 1350.

In sum, dehydrogenases, in particular members of the short-chain alcohol dehydrogenase family, were known, at the time the present application was filed, to exhibit similar biological activity even among members of this class whose overall sequence identity was low. Highly conserved "core" residues were known at the time to be located primarily at the N-terminal region and other selected regions of the molecule. The skilled artisan would have been able to generate dehydrogenase mutants within the scope of the claims by mutating residues outside the highly conserved amino acid stretches using routine molecular biology techniques. Such mutants could be tested using the assays described and exemplified in the instant specification.

Previously presented claim 37 and newly added claim 39 are directed to methods of producing an (S)-4-halo-3-hydroxybutyric acid ester derivative using an (R)-2-octanol dehydrogenase that is a variant of SEQ ID NO:2 with up to 50 and 30 conservative amino acid substitutions, respectively. As discussed during the interview, a conservative substitution is the replacement of one amino acid residue with a different one having a similar side chain (see specification at page 14, lines 15-26). For example, an amino acid having a basic side chain (e.g., lysine, arginine, histidine), acidic side chain (e.g., aspartic acid and glutamic acid), or beta-branched side chain (e.g., threonine, valine, isoleucine) is replaced with a different amino acid from the same group. *Id.* Given the similarities in the side chains, conservative substitutions are expected to have little, if any, impact on the activity of the dehydrogenase. Accordingly, Applicants submit that the specification fully enables the scope of claims 37 and 39.

In another aspect of the rejection, the Examiner acknowledges that "it is routine in the art to screen for multiple substitutions or multiple modifications as encompassed by the instant claims," but asserts the specific amino acid positions replacements that can be made within a protein are limited and the results of such modifications are unpredictable. In particular, the Examiner states that:

> [O]ne skilled in the art would expect any tolerance to modification for a given protein to diminish with each further and additional modification, e.g., multiple substitutions. (Office Action at page 7).

Applicants respectfully disagree with the Examiner's position. As mentioned above, dehydrogenases are a class of enzymes that appear to be particularly tolerant of amino acid substitutions. Similar results have been reported in the literature for other proteins (see, e.g., Bowie *et al.* (1990) *Science* 247:1306-10, page 1306, col.2, lines 12-13). For example, Bowie *et al.* cite as evidence a study carried out on the *lac* repressor. Of approximately 1500 single amino acid substitutions at 142 positions in this protein, about one-half of the substitutions were found to be "phenotypically silent:" that is, had no noticeable effect on the activity of the protein (page 1306, col. 2, lines 14-17). Presumably the other half of the substitutions exhibited effects ranging from slight to complete abolishment of repressor activity. Thus, one can expect, based on teachings by Bowie *et al.*, to find over half (and possibly well over half) of random substitutions in any given protein to result in mutated proteins with full or nearly full activity. These are far better odds than those at issue in *In re Wands,* 858 F.2d 731 (Fed. Cir. 1988), in which the Federal Circuit stated that screening many hybridomas to find the few that fell within the claims was not undue experimentation. Based on the teachings by Bowie *et al.*, one would predict that even random substitution of residues in SEQ ID NO:2 will predictably result in a majority of the mutants' having (R)-2-octanol dehydrogenase activity. Given the knowledge in the art about regions of sequence homology among dehydrogenases, one of ordinary skill would know to make changes preferentially in other regions, or only conservative changes in those regions, thereby making the predictability of success even higher than in the *lac* repressor study reported by Bowie *et al.* Furthermore, the specification amply teaches how to make and test mutants to find those with the activity required by the claims, as described above.

In view of the foregoing, reconsideration and withdrawal of this rejection is respectfully requested.

SUMMARY

No fee is believed to be outstanding at this time, but if necessary, apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 14879-090002.

The above rejections are either improper or do not pertain to the claims as newly amended and should be withdrawn. The present claims are in condition for allowance.

If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicant's Attorney at (617) 542-5070.

Respectfully submitted,

Date: August 3, 2006

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Appendix A

Blast 2 Sequences results

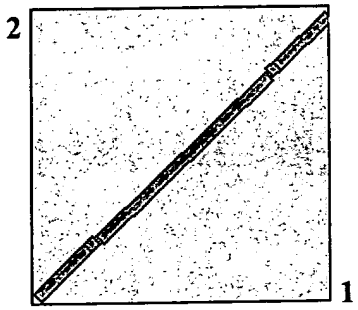
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.13 [Nov-27-2005]

Matrix BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.000 wordsize: 3 Filter ☐ View option Standard
Masking character option X for protein, n for nucleotide Masking color option Black
☐ Show CDS translation Align

Sequence 1: lcl|6706507SEQ ID NO:2
Length = 254 (1 .. 254)

Sequence 2: gi|2632694|gi|2632694glucose 1-dehydrogenase [Bacillus subtilis subsp. subtilis str. 168]
Length = 261 (1 .. 261)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

```
 Score =  191 bits (484),  Expect = 5e-47
 Identities = 107/249 (42%), Positives = 153/249 (61%), Gaps = 9/249 (3%)

Query  8   KVAVVTGALSGIGLSVAKKFLQLGAKVTISDVSGEKKYHETVV-ALKAQNLNTDNLHYVQ  66
           KV  +TGA SG+G  ++A +F +  AKV I+  S ++  +E      +KA          VQ
Sbjct  8   KVVAITGAASGLGKAMAIRFGKEQAKVVINYYSNKQDPNEVKEEVIKAGG----EAVVVQ  63

Query  67  ADSSKEEDNKKLISETLATFGGLDIVCANAGIGKFAPTHETPFDVWKKVIAVNLNGVFLL  126
            D +KEED K ++    +  FG LDI+  NAG+     P+HE P    W KVI   NL G FL
Sbjct  64  GDVTKEEDVKNIVQTAIKEFGTLDIMINNAGLENPVPSHEMPLKDWDKVIGTNLTGAFLG  123

Query  127 DKLAINYWLEKSKPGVIVNMGSVHSFVAAPGLAHYGAAKGGVKLLTQTLALEYASHGIRV  186
            + AI Y++E    G ++NM SVH   +  P   HY A+KGG+KL+T+TLALEYA  GIRV
Sbjct  124 SREAIKYFVENDIKGNVINMSSVHEVIPWPLFVHYAASKGGIKLMTETLALEYAPKGIRV  183

Query  187 NSVNPGYISTPLIDEV---PKERLDKLVSLHPIGRLGRPEEVADAVAFLCSQEATFINGV  243
            N++ PG I+TP+  E     PK++ D + S+ P+G +G  PEE+A  VA+L S+EA+++ G+
Sbjct  184 NNIGPGAINTPINAEKFADPKQKAD-VESMIPMGYIGEPEEIAAVAAWLASKEASYVTGI  242

Query  244 SLPVDGGYT  252
           +L  DGG T
```

Sbjct 243 TLFADGGMT 251

CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda	K	H
0.316	0.134	0.386

Gapped

Lambda	K	H
0.267	0.0410	0.140

Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Sequences: 1
Number of Hits to DB: 543
Number of extensions: 319
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's gapped: 1
Number of HSP's successfully gapped: 1
Length of query: 254
Length of database: 1,231,379,889
Length adjustment: 130
Effective length of query: 124
Effective length of database: 1,231,379,759
Effective search space: 152691090116
Effective search space used: 152691090116
Neighboring words threshold: 9
X1: 16 (7.3 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 41 (21.6 bits)
S2: 76 (33.9 bits)

Appendix B

New Enzymes for Organic Synthesis

Screening, Supply and Engineering

With Contributions by
F. H. Arnold, L. Elling, W. Hummel, J.-C. Janson, M. Kataoka, M. Kobayashi, J. C. Moore, J. Ogawa, M. Pohl, S. Shimizu, J. Thömmes

With 36 Figures and 30 Tables

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Appendix B

Lothar Elling

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: 79

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ıns. In: Dolphin D, Poulson R, al biochemical, medical aspects

(ed) Methods in Plant Biochem-

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ıs FA, Tanner W (eds) Encyclo-hydrates I Intracellular Carbo-

A, Adrian H, Kula M-R (1995)

A, Kula M-R, Elling L (1993)

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New Alcohol Dehydrogenases for the Synthesis of Chiral Compounds

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Dedicated to Professor Dr. Maria-Regina Kula on the occasion of her 60th birthday

The enantioselective reduction of carbonyl groups is of interest for the production of various chiral compounds such as hydroxy acids, amino acids, hydroxy esters, or alcohols. Such products have high economic value and are most interesting as additives for food and feed or as building blocks for organic synthesis. Enzymatic reactions or biotransformations with whole cells (growing or resting) for this purpose are described. Although conversions with whole cells are advantageous with respect to saving expensive isolation of the desired enzymes, the products often lack high enantiomeric excess and the process results in low time-space-yield. For the synthesis of chiral alcohols, only lab-scale syntheses with commercially available alcohol dehydrogenases have been described yet. However, most of these enzymes are of limited use for technical applications because they lack substrate specificity, stability (yeast ADH) or enantioselectivity (*Thermoanaerobium brockii* ADH). Furthermore, all enzymes so far described are forming (*S*)-alcohols. Quite recently, we found and characterized several new bacterial alcohol dehydrogenases, which are suited for the preparation of chiral alcohols as well as for hydroxy esters in technical scale. Remarkably, of all these novel ADHs the (*R*)-specific enzymes were found in strains of the genus *Lactobacillus*. Meanwhile, these new enzymes were characterized extensively. Protein data (amino acid sequence, bound cations) confirm

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that these catalysts are novel enzymes. (*R*)-specific as well as (*S*)-specific ADHs accept a broad variety of ketones and ketoesters as substrates. The applicability of alcohol dehydrogenases for chiral syntheses as an example for the technical use of coenzyme-dependent enzymes is demonstrated and discussed in this contribution. In particular NAD-dependent enzymes coupled with the coenzyme regeneration by formate dehydrogenase proved to be economically feasible for the production of fine chemicals.

Abbreviations

ADH = alcohol dehydrogenase
YADH = yeast alcohol dehydrogenase
HLADH = horse liver alcohol dehydrogenase
TBADH = *Thermoanaerobium brockii* alcohol dehydrogenase
FDH = formate dehydrogenase

-specific ADHs accept a broad
of alcohol dehydrogenases for
-dependent enzymes is demon-
ndent enzymes coupled with the
: economically feasible for the

irogenase

1 Introduction

1.1 Dehydrogenases as Catalysts for the Synthesis of Chiral Compounds

Enzymes are attractive catalysts for organic synthesis. They are highly selective and specific and many problems of chemical synthesis such as isomerization, racemization or rearrangement can be avoided because of the mild enzymatic reaction condition. Particularly for asymmetric syntheses, enzyme catalyzed reactions offer notable advantages for the organic chemist. Considering the six main enzyme groups according to the classification of the International Union of Biochemistry [1] the oxidoreductases, hydrolases and lyases represent the most useful enzymes. Hydrolases and lyases are catalysts which in general do not need additional cofactors or coenzymes which simplify the process engineering and optimization of the reaction. Oxidoreductases require coenzymes which complicates the process engineering and tends to increase the product costs; coenzymes are too expensive to provide them in stoichiometric amounts and efficient steps for their regeneration are required.

During the last years, enzyme-catalyzed syntheses had been developed which demonstrate the applicability of such methods. Lipases and esterases are well established tools to produce chiral materials. Processes using coenzyme-dependent enzymes were developed during the last years for the preparation of optically active hydroxy and amino acids employing commercially available enzymes such as lactate dehydrogenases or new dehydrogenases such as *L*- or *D*-hydroxyisocaproate dehydrogenases or *L*-phenylalanine dehydrogenase (Review [2–4]).

Enzyme-catalyzed syntheses of enantiomeric pure hydroxy acids use lactate dehydrogenases (LDH; E.C. 1.1.1.27) or hydroxyisocaproate dehydrogenases (HicDH). Both kinds of enzymes are available as *D*- or *L*-specific catalysts. *L*-specific [5, 6] LDHs as well as *D*-specific [7–10] LDHs favorably catalyze the reduction of pyruvate, HicDHs (and mandelate dehydrogenase, too) convert keto acids with longer aliphatic or aromatic side chains. These enzymes can be isolated from *Lactobacillus* strains [11–14].

Reductive amination reactions of keto acids are performed with amino acid dehydrogenases. NAD-dependent leucine dehydrogenase from *Bacillus* sp. is of interest for the synthesis of (*S*)-*tert.*-leucine [15–17]]. This chiral compound has found widespread application in asymmetric synthesis and as a building block of biologically active substances. The enzyme can also be used for the chemoenzymatic preparation of (*S*)-hydroxy-valine [18] and unnatural hydrophobic branched-chain (*S*)-amino acids. NAD-dependent *L*-phenylalanine dehydrogenase from *Rhodococcus* sp. [19] has been used for the synthesis of *L*-homophenylalanine ((*S*)-2-Amino-4-phenylbutanoic acid) [9]. These processes with water-soluble substrates and products demonstrate that the use of coenzymes must not

be a limiting component, the coenzymes are stable enough for long-term applications and using an efficient coenzyme-regeneration step and an appropriate reaction engineering it is possible to reach sufficient turnover numbers for the coenzyme (significantly more than 100,000 are obtainable) that such reactions become economically feasible.

Chiral alcohols are valuable products mainly as building blocks for pharmaceuticals or agro chemicals or as part of chiral catalysts. Cheap biotransformation methods for the selective reduction of particular ketone compounds are known for many years rather catalyzed by fermentation than with isolated enzymes. Products prepared with whole cells such as baker's yeast often lack high enantioselectivity and there were several attemps to use isolated enzymes. Resolution of racemates with hydrolases are known in some cases but very often the reduction of the prochiral ketone using alcohol dehydrogenases are much more attractive.

1.2 Prerequisites for the Use of Dehydrogenases

Alcohol dehydrogenases are enzymes that are well known from physiological and biochemical studies on the primary metabolism of cells. Several ADHs are commercially available and for some of them such as the ADH from yeast or liver details concerning the structure and reaction mechanism have been elucidated. For preparative applications however they seldom meet the requirements and new enzymes are needed for this field.

Several prerequisites have to be fulfilled to make an efficient application of these enzymes possible:

– Enzymes Should Work with High Selectivity and High Yield

In the use of whole cells severe problems may arise from strain specific activity of intracellular enzymes reacting with the product. For instance, the organism may use the substrate or the product as the carbon source or intracellular esterase activities may influence the yield of hydroxy esters formed by enzymatic reduction of keto esters. These problems may be avoided using isolated enzymes. These potential side reactions also define the purification grade of a technical enzyme sample because the complete separation of the disturbing activities must be ensured.

– Enzymes Should Work with High Enantioselectivity and Predictions About Their Stereospecificity Should be Possible

Although conversions with whole cells save expensive procedures to isolate the desired enzymes, the products however often lack sufficient enantiomeric excess

and the predicition of the stereospecificity of the reaction is difficult. In most cases processes with whole cells result only in low space-time-yield.

Because of its availability, baker's yeast is by far the most widely used biological catalyst for enantioselective reduction reactions [20–25]. Actually it was applied to nearly countless syntheses of β-hydroxyesters [26, 27]. The results demonstrate that the enantioselectivity of yeast catalyzed reductions of β-ketoesters depends on the structure of the substrate. Along a homologous series of C_1- to C_{12}-esters of 4-chloroacetoacetic acid, a shift in the stereochemistry of the alcohol produced was observed from the (*S*)-hydroxyester with an ee = 0.7% using the C_1-(methyl)-group to the (*R*)-hydroxyester with an ee = 95% containing the octyl-group [25]. Detailed biochemical characterization of the enzymes involved revealed that baker's yeast does possess three enzymes capable to reduce β-ketoesters, none of which however is the commercially available yeast ADH, which is thus obviously not involved in such reactions. Two of the isolated enzymes are (*R*)- and one is (*S*)-specific. The substrate specificity of these enzymes shows that in increasing the chain length in the ketone led to contrary reaction rates of the three enzymes, thus producing often a mixture of isomers. Therefore it is not possible to predict the stereospecificity of a reaction using a new substrate or a new yeast strain. Otherwise, the enantioselectivity can be altered either by using mutants [28] or various strains of baker's yeast [29–31] or by inhibiting one or several of these enzymes. Addition of allyl alcohols [32] or methyl vinyl ketone [33] supports the formation of (*R*)-products, presumably by inhibiting an (*S*)-specific dehydrogenase, but in some cases, results in low yield [34]. Immobilization of the cells [35, 36] or variation of medium constituents may also influence the ratio of isomers, e.g. addition of ammonium salts (53), thiamine (55) or oxygen supply (51.52).

Using isolated enzymes instead of whole cells, similar problems are to be considered only in a few cases. ADH from *Thermoanaerobium brockii* shows varying enantiomeric excess of the product depending on the structure of the ketone to be reduced. Conversions with this enzyme yield in products with low (20% for the reduction of acetophenone) or high ee value (100% for the reduction of *p*-Cl-acetophenone). Predictions about the stereospecificity of HLADH catalyzed reductions can be made for simple acyclic substrates applying Prelog's rule [37] and for more complex compounds using the cubic-space model developed by Jones and Jakovac [38].

– Efficient Coenzyme-Regeneration Methods Should be Available

Using whole cells for preparative conversions, the metabolic status of the cells is of importance for the efficiency of the regeneration step. This concerns both the available intracellular concentration of the coenzyme and the kind of regeneration. In particular NADP-dependent enzymes may be limited by the level of this coenzyme taking into consideration the Km-values of the dehydrogenases for

the coenzymes and the concentrations which are necessary for maximal activity. From results with yeast strains it is likely that the intracellular concentrations of nicotinamide coenzymes differ in strains of various origins [39].

Isolated oxidoreductases always depend on cofactors for the transfer of electrons. Enzyme groups which are well characterized with respect to their biochemistry are those requiring the nicotinamide coenzymes NAD or NADP, the flavins FAD or FMN and the ortho-quinoids such as pyrroloquinoline quinone (PQQ) or trihydroxy-phenylalanine (TOPA).

Among these cofactor-dependent enzymes, the NAD(P)-dependent are of central interest. Due to their high prices, the required coenzymes cannot be applied in stochiometric amounts but must be efficiently regenerated if such enzymes are to be employed as catalysts for preparative chemistry. Generally there are two approaches possible: the application of a second enzyme, or a second substrate. So far, only the use of formate dehydrogenase from *Candida boidinii* for the regeneration of NADH is demonstrated in technical scale. Alternate concepts are discussed such as the application of metallorganic compounds or a modified formate dehydrogenase obtained by protein engineering.

- Enzymes Should be Available in Technical Scale

Several methods for the production and isolation of extra- and intracellular enzymes from wild-type strains are established during the last years. Highly efficient methods for the scale-up of dehydrogenases have been developed for the enzyme formate dehydrogenase [40, 41]. Alternately, gene cloning can be used successfully to obtain an efficient yield of the desired catalyst.

- Enzymes Should Convert Hydrophobic Substrates at an Efficient Space-Time-Yield

Most of the substrates or products which are interesting for organic chemistry are scarcely soluble in aqueous solutions. Unfortunately, enzymes are often deactivated when used in organic solvents. Successful conversions of the hydrophobic keto compounds thus can demonstrate general reaction principles for enzyme-catalyzed transformations of hydrophobic substrates. Various concepts concerning the application of enzymes for the transformation of hydrophobic compounds have been developed, but none of them have been applied so far in technical scale.

- Enzymes can be Modified by Protein Engineering

In order to overcome limitations derived from the protein structure such as lacking stability, substrate or coenzyme specificity, protein engineering methods

can be applied. Some examples are published which describe specific mutations of dehydrogenases.

This article will focus on new developments on the field of NAD(P)-dependent dehydrogenases, in particular on new alcohol dehydrogenases applicable for preparative chemistry. Biochemical properties of dehydrogenases useful for the synthesis of chiral hydroxy esters and alcohols are summarized in this contribution with respect to more recent studies on enantioselective reduction of prochiral ketones and to new enzymes for this purpose. Reviews covering earlier works in this field can be found in [42–44].

2 Alcohol Dehydrogenases

2.1 Screening and Site-Directed Mutagenesis for the Development of New Enzymes

Even though there is a great number of known enzymes depending on nicotinamide coenzymes, it seems sensitive for several reasons to screen for novel catalysts with respect to their technical applications. The growing importance of chiral compounds [45–47] demands new enzymes with new substrate specificities. The commercially available enzymes show limitations such as the insufficient operational long-term stability, lack of activity in organic solvents or limited substrate acceptance. The development of highly efficient methods for the regeneration of coenzymes such NADH or NADPH, however, facilitates syntheses that are inexpensive respecting the cofactor involved.

New or improved catalysts can be obtained by two basically various methods: screening for enzymes among naturally available sources or site-directed mutagenesis of known enzymes. Screening procedures with microorganisms, plants or animal cells represent the traditional way to discover new enzymes. Microorganisms are of particular interest because of their short generation time and the large diversity of metabolic pathways and enzymes involved. Special techniques with enrichment cultures or rapid assay methods supported by automation or miniaturization are useful to detect new enzyme producers. However, there are only a few generally applicable screening methods and only a few review articles available on this field [48–51], because successful screening methods essentially depend in detail on the problem to be solved. One strategy to obtain new or improved enzymes is given in Figure 1.

Literature on microbial physiology and biochemistry may be helpful for the choice of strains to find enzymes for the biotransformation of naturally occurring substances. Screening of enzymes to convert unnatural compounds however usually implies an accidental selection of microorganisms. Actually, even such a preliminary approach gives valuable informations about the occurrance of an enzyme activity.

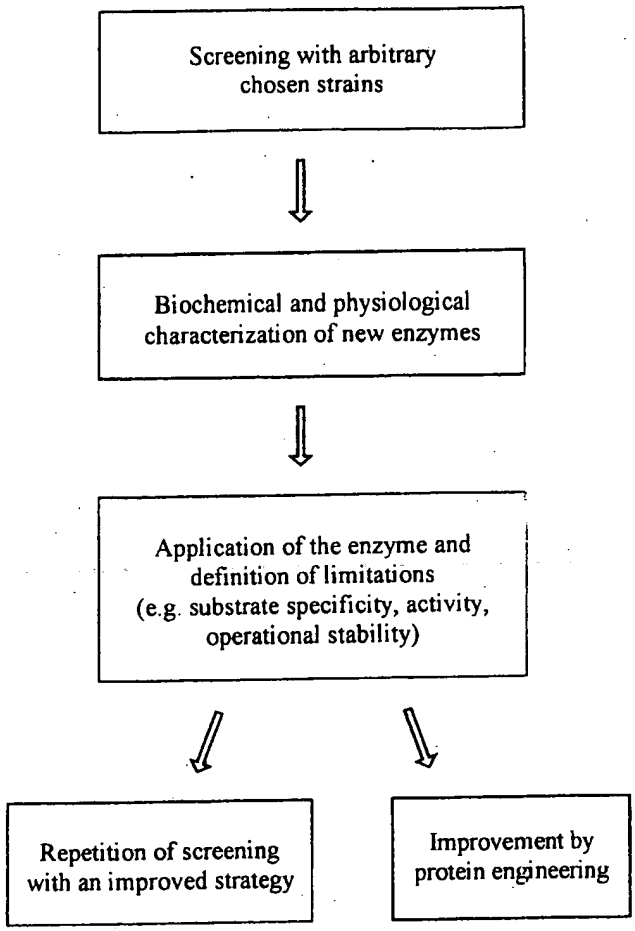

Fig. 1. Screening strategies to obtain new or improved enzymes by screening.

Several new enzymes which proved to be useful as technical catalysts were discovered by screening natural habitats. Some examples of NAD(P)-dependent enzymes may demonstrate the various screening procedures: Bacteria with NAD-dependent L-phenylalanine dehydrogenase were isolated from soil samples by providing the culture medium with L-phenylalanine [19, 52–54]. An NAD-independent trimethylamine dehydrogenase could be obtained using enrichment cultivation techniques in a chemostat [55]. A mutant strain of *Rhodobacter sphaeroides* producing a new galactitol dehydrogenase was isolated from a chemostat culture [56]. Strains of *Thermoanaerobium brockii* that produce a thermostable NADP-dependent alcohol dehydrogenase were isolated from a hot spring site in Yellowstone Park [57]. Further thermostable alcohol dehydrogenases could be obtained from a novel strain of *Bacillus stearothermophilus*

growing at 70° C [58] and from a novel archaebacterium *Sulfolobus solfataricus* [59, 60]. Enrichment of microorganisms culturing them on selected alcohols as the carbon source resulted in a new NAD-dependent secondary alcohol dehydrogenase with (*R*)-selectivity for the alcohol [61, 62].

Further improvements to an enzyme can be made by testing related strains in order to find enzyme variants. No general rule exists about the distribution of certain enzymes within microbial groups; some enzymes such as lactate dehydrogenase occur in all organisms of a family (*Lactobacillaceae* in this case), others such as leucine dehydrogenase or alanine dehydrogenase are found in all strains of a genus (*Bacillus*) and some seem to occur only in a single strain of a species. Phenylalanine dehydrogenase for instance occurs in *Rhodococcus erythropolis* and in strains of a new species of *Rhodococcus* [19], but it does not in other *Rhodococcus* species [54]. Obviously, this is not due to the sample preparation or the assay, because the occurrence of phenylalanine dehydrogenase is correlated with the ability to degrade of *L*-phenylalanine and the inactive species of *Rhodococcus* were found to be unable to catabolize phenylalanine.

To know about the distribution of enzymes within the microbial world represents a valuable information in developing and improving a technical enzyme because this offers the chance to obtain enzyme variants with seperate biochemical properties. Oligo-1,6-glucosidases for instance, from strains of five various *Bacillus* species showed an increase in thermostability from mesophile, facultative thermophile, obligate thermophil, up to extreme thermophile, which corresponds with the proline content of the enzymes [63], although they are homologous proteins with respect to their catalytic and molecular properties. These enzyme variants also show various stabilities against organic solvents or denaturing concentrations of salt, and various ranges of pH-stability.

Hydroxyisocaproate dehydrogenases (HicDHs) are enzymes that reduce 2-keto acids with branched-chain or aromatic side chains enantioselectively to the corresponding hydroxy acids. They occur in strains of various species of *Lactobacillaceae* and were originally found in the course of a screening for enantioselective 2-hydroxy acid dehydrogenases [11–13]. For lactate dehydrogenases exhibit a limited substrate specificity, these alternative enzymes with their broad substrate acceptance are important for preparative purposes. The physiological significance of HicDHs are not yet clear, but such an activity could be demonstrated in several *Lactobacillus* strains. The biochemical characterization of HicDHs from various strains of *Lactobacillaceae* reveal separate enantioselectivities and kinetic properties. Most of the enzymes yield *D*-hydroxy acids exept the one from *Lactobacillus confusus* which is *L*-specific. The selectivities for keto acids are quite different, e.g. the enzyme from *Lactobacillus casei* DSM 20 008 reveals a high affinity (low K_m-value) for ketoisocaproate compared to the structurally related compounds ketomethylvalerate and ketoisovalerate. Significantly, the enzyme from this strain is the only one which shows such a high selectivity for ketoisocaproate, whereas another strain of this species, *L. casei* DSM 20 244 accepts the three keto acids with almost identical affinity.

These few examples demonstrate that at least some optimization of an enzyme, designed as a technical catalyst, can be provided by enzyme variants obtained by repeated screening steps. Basing upon biochemical and physiological data from preliminary enzyme characterization studies such improvements may be obtained by changing the screening procedure. An example for the improvement of an (*R*)-specific alcohol dehydrogenase is given below. Variants of this alcohol dehydrogenase with a significantly higher thermostability could be detected by an antibody-supported screening method.

Site-directed mutagenesis is a new and rapidly developing approach for the rational modification of an enzyme. This method requires exact information about the amino acid sequence, the three-dimensional structure and the active site of the enzyme. An example for a successful site-directed mutagenesis is represented by *L*-lactate dehydrogenase, which could be converted into an *L*-malate dehydrogenase by replacing Gln-102 to Arg [64]. The substrate specificity of lactate dehydrogenase from *Bacillus stearothermophilus* was extended by site-directed mutagenesis as well [65]. In some cases, the coenzyme specificity could be altered. Naturally, most of the NAD(P)-dependent dehydrogenases are highly selective for only one of the coenzymes. Some successful approaches were reported to change or extend this coenzyme specificity: The NADP-dependent glutathione reductase was altered into a NAD-specific enzyme by site-directed mutagenesis [65], and glyceraldehyde 3-phosphate dehydrogenase which depends on NAD was changed by two mutations into an enzyme accepting NADP, too [66].

Formate dehydrogenase is an important means for the regeneration of NADH. All formate dehydrogenases described so far in the literature are highly specific for NAD. Only very recently, the coenzyme specificity of the formate dehydrogenase from a *Pseudomonas* strain was altered to accept both NAD and NADP [67–69]. The preferred substrates of wild-type aspartate aminotransferase are the anionic amino acids L-aspartate and L-glutamate. This specificity was changed by the replacement of the active-site Arg by Asp, which generates an enzyme accepting the cationic amino acids L-lysine and L-arginine [70]. In the active site of yeast alcohol dehydrogenase, two amino acids with bulky side chains, Trp-93 and Thr-48 were replaced by Phe and Ser, respectively. This facilitates the oxidation of long-chain alcohols such as propanol, butanol, pentanol, hexanol, heptanol, octanol and cinnamyl alcohol [71].

Besides these rather complex coenzyme-dependent enzymes, the none-coenzyme requiring protease subtilisin is the most extensively mutated enzyme. The substrate specificity of the enzyme as well as its dependence on pH and its stability were altered by site-directed mutagenesis [72–78]. As the knowledge about exact details of the structure and active site of the enzyme is essential for the application of this method, progress in this field is difficult to achieve. Site-directed mutagenesis as a means of catalyst improvements will be used only after extensive application of conventional optimization procedures.

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2.2 Biochemical Properties of Alcohol Dehydrogenases

2.2.1 Classification of NAD(P)-Dependent Alcohol Dehydrogenases

Alcohol dehydrogenases can be subdivided with respect to various criteria, two of which are in particular relevant for the application of these enzymes: first structural and protein chemical data, especially subunit size and occurrence of metal ions, and second the stereochemical course of the catalyzed reaction and the consequential chirality of the formed alcohol.

Alcohol dehydrogenases are in generally subdivided into three groups [79], the medium-chain, zinc-containing ADHs, represented by horse liver ADH, short-chain ADHs without any metal ion, represented by the *Drosophila* ADH and the "iron-activated" long-chain ADHs with the ADH-II from *Zymomonas mobilis* as the typical enzyme of this group. A recent review concerned with the molecular characterization of microbial ADHs is given by Reid and Fewson [80]. Originally, the classical yeast and liver ADHs had been termed as the long-chain ADHs in contrast to the short-chain enzymes, but were renamed as the medium-chain family [81, 82] after the still-longer ADHs had been discovered. Table 1 summarizes characteristic biochemical and microbiological data of enzymes of these groups.

Table 1. Biochemical and microbiological characterization of alcohol dehydrogenases (Classification according to [79])

Parameter	Short-chain ADH	Medium-chain ADH	Long-chain ADH, iron-activated
Amino acids per subunit	approx. 250	approx. 350	approx. 385
Metal requirements	no requirements	Zn^{2+}	Fe^{2+}
Occurrence	prokaryotes *Drosophila* ssp. mammalian tissues	pro- and eukaryotes microbes, plants, mammalian tissues	prokaryotes *Saccharomyces cerevisiae* (ADH IV)
Enzyme (Ref.)	Sorbitol dehydrogenase [83]	Benzyl-ADH [84]	Glycerol dehydrogenase [85]
(Organism)	(*Rhodobacter sphaeroides*)	(*Acinetobacter calcoacticus*)	(*Bacillus stearothermophilus*)
	LKADH [86] (*Lactobacillus kefir*)[a]	TBADH [87, 88] (*Thermoanaerobium brockii*)	Propandiol dehydrogenase [89] (*E. coli*)
	LBADH [90] (*Lactobacillus brevis*)[a]	ADH (Isoenzymes I-III) [91] (*Saccharomyces cerevisiae*)	
		HLADH [92] (horse liver)	
		Sorbitol dehydrogenase [93] (sheep liver)	
		sec.-ADH [94] (*Rhodococcus erythropolis*)	

[a] According to their amino acid content and structural relationship (see below), these enzymes are short-chain dehydrogenases, although they contain Zn^{2+} and require Mg^{2+} for their activity.

The medium-chain ADHs are defined basing upon the classical liver alcohol dehydrogenase (HLADH), which is now known to represent a large family of enzymes [82, 95], which contain frequently but not always [96] zinc at the active site. Medium-chain ADHs exist in dimeric or tetrameric forms, and depend on NAD or NADP, respectively. Alignment of amino acid sequences revealed that proteins other than ADHs are related to these enzymes, for example, threonine dehydrogenase from *E. coli* [97], human sorbitol dehydrogenase [98], glucose dehydrogenase from *Thermoplasma acidophilum* [99], or reductases such as rat [100] or yeast enoyl reductase [101] and *E. coli* quinone oxidoreductase are related to this kind of ADHs. An alignment of 106 proteins of this large enzyme family [82] revealed that only three residues are strictly conserved, which are all glycines. These residues are located in both domains and thus do not only constitute the coenzyme binding motif.

The tertiary structure of HLADH, the representing enzyme of this group, was refined to 2.4 Å resolution with X-ray diffraction [102–105], and the three-dimensional modeling of other medium-chain ADHs is based upon this structure [106, 107]. The tertiary structure of yeast ADH was found to be similar to that of HLADH although the primary structure of YADH is quite different.

The second large subgroup consists of the short-chain alcohol dehydrogenases, which have been attracting new attention in the last years because many enzymes were found to belong to this family. It was firstly defined [108, 109] with regard to the structures of *Drosophila* ADH [110, 111]], ribitol dehydrogenase from *Enterobacter aerogenes* [112, 113], and glucose dehydrogenase from *Bacillus megaterium* [114, 115]. Meanwhile more than 20 pro- and eukaryotic proteins are described to belong to this family [80]. Dimeric as well as tetrameric proteins were found, but not all these enzymes are actually alcohol dehydrogenases. Hydroxysteroid dehydrogenases, polyol dehydrogenases and proteins the biochemical function of which is not known, belong to this group, too. The enzymes may depend on NAD as well as on NADP. However related with respect to their subunit size and coenzyme binding motif, all these enzymes are very dissimilar regarding the residue identities of 15–35% only. Proteins which belong to the short-chain ADHs are not as well characterized as the medium-chain dehydrogenases. The secondary structure of the *Drosophila* ADH was predicted by Thatcher and Sawyer [110] who suggested an alternating βαβ region (a nucleotide-binding domain) at the N-terminus of the protein. This assumed function of the N-terminal part of these enzymes was supported by a conserved glycine-rich domain (GXXXGXG) found in most of the short-chain dehydrogenases.

The recent elucidation of a large number of short-chain ADH sequences permits a detailed characterization of this enzyme group. As many as six domains are found to be significantly conserved. A domain located at the N-terminus with a length of approximately 30 amino acids is generally assumed to be the coenzyme binding site. A second conserved domain is a hydrophobic region comprising 10 or 11 residues, respectively. A further conserved domain

consists of 18 amino acids are probably involved in the dimerization of enzyme subunits or facilitate the hydride transfer. It starts with a totally conserved tyrosine and ends with a highly conserved acidic amino acid.

The third subgroup of alcohol dehydrogenases consists of "iron-activated" enzymes. The first enzyme detected to belong to this group was the ADH II from *Zymomonas mobilis* [116] followed by the observation that the ADH IV from *Saccharomyces cerevisiae* shows more than 50% identity to this bacterial ADH [117]. No data about the secondary or tertiary structures of the enzymes in this subgroup are available currently. A prediction based upon the Chou and Fasman analysis [118] indicates that these enzymes are rich in α-helices.

For a classification of the ADHs according to the stereochemistry of the reduction one has to consider that the reduced coenzymes NADH and NADPH possess two diastereotopic hydrogens, pro-*R* (A-specific dehydrogenases) and pro-*S* (B-specific enzymes) and that the substrate ketone can be attacked by two sides. It is possible to determine which hydrogen is transferred by the enzyme using [4-^{2}H]-labelled NAD(P)H for the reduction reaction or labelled alcohol and NAD(P) [119, 120]. This hydrogen can attack the carbonyl group from the *re*- or the *si*-side, resulting in an (*S*)- or (*R*)-alcohol. Table 2 shows the various stereochemical courses of the hydrogen transfer and representative enzymes for the different cases. Most alcohol dehydrogenases (sometimes termed as A-enzymes) transfer the pro-*R* hydrogen to the *re*-face of substrate ketone forming (*S*)-alcohol, a process characterized by Prelog's rule [37]. Table 2 also illustrates that neither the kind of hydrogen transfer nor the stereochemistry of the carbonyl reduction is correlated to a preference of the kind of coenzyme (NAD or NADP) involved.

Table 2. Stereochemistry of the hydrid transfer catalyzed by alcohol dehydrogenases

Parameter	Enzyme group			
	E_1	E_2	E_3	E_4
Transferred hydrogen	pro-*R*	pro-*S*	pro-*R*	pro-*S*
Face of the attacked carbonyl	*si*-face	*si*-face	*re*-face	*re*-face
Chirality of the formed alcohol	(*R*)-alcohol	(*R*)-alcohol	(*S*)-alcohol	(*S*)-alcohol
Alcohol dehydrogenases (Coenzyme)	*Lactobacillus kefir* (NADP) *Pseudomonas* sp. (NAD)	*Mucor javanicus* (NAD)	yeast (NAD) horse liver (NAD) *Thermoanaerobium brockii* (NADP)	not known

2.2.2 *Characterization of Available Alcohol Dehydrogenases*

Many NAD(P)-dependent alcohol dehydrogenases from microorganisms, plant and mammalian cells have been purified and biochemically characterized so far. Most of them may be classified into three subgroups, and they represent a large database to give information about phylogenetic and evolutionary relationships and to allow comparative biochemical studies on the function of conserved sequences and structure-function relationships. Only few of these enzymes are important for preparative applications, particularly those that are commercially available or easy to prepare. Among the commercially available ADHs, there are some like the hydroxysteroid dehydrogenases or glycerol dehydrogenase that show a very narrow substrate specificity. Nevertheless, the preparation of some chiral alcohols in the gram-scale has already demonstrated the applicability of these enzymes for a limited number of substrates. Besides these specific enzymes, there are three alcohol dehydrogenases, isolated from horse liver (HLADH), yeast (YADH) and *Thermoanaerobium brockii* (TBADH) that are important because they accept a broad spectrum of substrates or are available at a very low price (Table 3).

Horse Liver ADH

Horse liver ADH is very useful for preparative applications. It is one of the oxidoreductases, which have been studied most exceedingly. It is a dimer consisting of nearly identical subunits. The subunits are designated as E (for

Table 3. Characterization of commercially available alcohol dehydrogenases which accept a rather broad spectrum of substrates

	Yeast ADH	Horse liver ADH	*T. brockii* ADH
Classification	medium-chain dehydrogenase	medium-chain dehydrogenase	medium-chain dehydrogenase
Required coenzyme	NADH	NADH	NADPH
Preferred substrates	aldehydes	aldehydes, cycl. ketones	ketones
Specific activity (U/mg)	300[a]	1–2[a]	30–90[b]
Enzyme costs ($/1000 Unit) (Sigma catalogue 1996)	1.0	530	870
Stability	sensitive to O_2	stable	stable
Limitations	low stability		low enantio-selectivity for some substrates; NADP-dependence; costs

[a] Oxidation of 1 µmol ethanol per min
[b] Oxidation of 1 µmol isopropanol per min

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	ketones
	30–90[b]
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	stable
	low enantio-selectivity for some substrates; NADP-dependence; costs

ethanol active) with 374 amino acids, and S (for steroid active), which differs from the E unit in only a six amino acid residue [103, 105]. There are three possible species of the enzyme, EE, SS and ES, however the EE form is dominating in commercially available highly purified products. Each subunit contains two zinc atoms. The X-ray structure was obtained with the EE isozyme.

HLADH converts a wide range of substrates. For the predicition of the stereoselectivity of reduction reactions, originally Prelog's diamond lattice model was applied, which is based upon the characteristic properties of the ADH of *Curvularia falcata* [37]. This model describes the stereospecificity of HLADH catalyzed reductions of simple acyclic substrates such as aldehydes. Later on, for more complex acyclic and cyclic substrates, a cubic-space model of the active site was developed [38, 121]. Other models are based upon symmetric properties [122–125] or upon a refined diamond lattice model [126–129].

Yeast ADH

Yeast ADH is a tetramer containing varying amounts of zinc. Although the amino acid sequence is quite different from HLADH, tertiary structures seem to be similiar in both enzymes [103, 105, 130, 131]. Yeast ADH has a very narrow substrate specificity and usually accepts aldehydes and methyl ketones, only [132, 133]. Compared to the other commercially available ADHs YADH is a cheap enzyme; but it is quite unstable. Thus, YADH is only of limited use for the preparation of chiral alcohols. Because of its low cost, YADH has been studied thoroughly regarding its potential for the regeneration of the reduced coenzymes [134–137], but ethanol being the regeneration substrate as well as acetaldehyde being the product deactivate YADH at very low concentrations.

Thermoanaerobium brockii ADH

TBADH is an NADP-dependent dehydrogenase which oxidizes preferably secondary alcohols. It was isolated from a thermophilic microorganism and exhibits a remarkable thermostability [138–140]. The enzyme remains stable up to 65°C. Since neither YADH nor HLADH are able to convert open-chain secondary alcohols, TBADH fills this gap: its reactivity is highest against secondary alcohols, and decreasing against linear and cyclic ketones and being low against primary alcohols. Because of this substrate spectrum, the stereospecific reduction of ketones can be coupled with the TBADH-catalyzed oxidation of isopropanol to regenerate NADPH. The enantiomeric excess of the product alcohols obtained by reduction with TBADH decreases if small substrates like methylethylketone are converted. Table 3 summarizes basical properties of the three commercially available alcohol dehydrogenases that are important for preparative applications.

Glycerol Dehydrogenases

Purified glycerol dehydrogenases are commercially available from several microbial sources, for instance *Bacillus megaterium* (NAD-dependent; 20 U/mg (glycerol oxidation)), *Cellulomonas* sp. (NAD; 50–125 U/mg), *Enterobacter aerogenes* (NAD; 25 U/mg) or *Aspergillus niger* (NADP; 10–20 U/mg), respectively. *In vivo*, these enzymes catalyze the interconversion of glycerol and dihydroxyacetone, *in vitro* they have been used for the enantioselective reduction of achiral 2-hydroxy ketones to chiral 1,2-diol, the kinetic resolution by reduction of racemic 2-hydroxy ketones and the stereoselective oxidation of meso-1,2-diols to 2-hydroxy ketones [141]. The most important application of glycerol dehydrogenases is the enzyme-catalyzed reduction of prochiral hydroxy ketones, whereas the oxidation reactions are limited by a severe product inhibition. Reduction of 1-hydroxy-2-propanone or 1-hydroxy-2-butanone to the corresponding (*R*)-diols are described with ee-values of 95–97% on a gram-scale [141].

Since glycerol dehydrogenase contains autoxidizable thiol groups, it is nessecary to perform these reaction in an inert atmosphere; antioxidants such as dithiothreitol or mercaptoethanol deactivate the enzyme at higher concentrations and should be avoided in preparative applications.

Hydroxysteroid Dehydrogenases

Several hydroxysteroid dehydrogenases (HSDHs) are available with various regioselectivities. They are named by their ability to oxidize or reduce various hydroxylated steroid structures, for instance 3α-hydroxysteroid dehydrogenase oxidizes androsterone at the 3α position. It can be isolated from *Pseudomonas testosteroni*. Commercially available samples with a specific activity of 15–50 U/mg are free of 3β-hydroxysteroid dehydrogenases. Other commercially available enzymes belonging to this group are 7α-HSDH from *Escherichia coli* or *Pseudomonas* sp., 12α-HSDH from *Bacillus sphaericus*, and 3α,20β-HSDH (cortison reductase) from *Streptomyces hydrogenans*. Keto groups of steroid and bile acid molecules can be reduced on a preparative scale regio- and stereospecifically using hydroxysteroid dehydrogenases [142–144]. The transformations are complete and virtually pure products are obtained. Reactions with neutral steroids, which are poorly soluble in aqueous buffers, are carried out in water-organic solvent two-phase systems [143]. Some of these enzymes catalyze transformations of non-steroid ketones, too. In particular, 3α-HSDH appears to be useful in oxidizing aromatic *trans*-diols with high enantioselectivity. All the commercially available HSDHs are rather expensive.

Diketone and Other Ketone Reductases from Microbial Sources

Diacetyl reductase (acetoin dehydrogenase) isolated from *Lactobacillus kefir* converts the prochiral diacetyl into optically pure (+)-acetoin (ee > 94%) [145].

This NAD-dependent enzyme was purified up to a specific activity of 1060 U/mg (diacetyl as substrate). The enzyme is stable at 57°C for 10 min, the temperature optimum is at 70°C. Besides diacetyl several other diketones were reduced.

From *Candida parapsilosis* an NADP-dependent carbonyl reductase was purified which can reduce a variety of diketones such as indole-2,3-diones and analogues, or dihydro-4,4-dimethyl-2,3-furandiones [146]. The reduction of the latter compound gives (*R*)-(-)-dihydro-3-hydroxy-4,4-dimethyl-2(3*H*)-furanone, a key intermediate in the synthesis of D-pantothenic acid. The reduction of 2-(6-carbomethoxyhexyl)cyclopentane-1,3,4-trione results in important intermediates of (-)-prostaglandin E_1 and (-)-prostaglandin E_2. An NAD-dependent carbonyl reductase from *Candida parapsilosis* converts a broad variety of carbonyl compounds resulting in (*S*)-hydroxy compounds [147, 148]. A carbonyl reductase from *Mucor ambiguus* is strictly specific for conjugated polyketone compounds only [149]. Similar enzymes seem to be widely distributed among microorganisms [146, 150–152]. Out of all these enzymes, only diacetyl reductase from *Lactobacillus kefir* is commercially available.

ADH from *Sulfolobus solfataricus*

An NAD-dependent alcohol dehydrogenase was isolated from the extreme thermophilic archaebacterium *Sulfolobus solfataricus* [59]. The enzyme has a broad substrate specificity that includes linear and branched primary alcohols, linear and cyclic secondary alcohols and linear and cyclic ketones. The enzyme is a dimer with a molecular weight of 37.6 kDa, it contains four zinc atoms per dimer. The gene encoding this ADH has been isolated and the primary structure, determined by peptide and gene analysis consists of 347 amino acids [60]. Due to these structural features it belongs to the medium-chain ADH family. The enzyme activity increases with temperature up to 95°C. At 60°C and 70°C the half-life was 20 h and 5 h, respectively. The specific activity of the highly purified enzyme is in the range of 3.9 U/mg, measured with benzyl alcohol at 65°C. *Sulfolobus* ADH is not available commercially.

Enoate Reductases

Enoate reductase [153, 154], which occurs in strains of *Clostridium* or *Proteus*, and 2-oxo-acid reductase [155] from *Proteus vulgaris* or *P. mirabilis* catalyzes the stereospecific reduction of substrates performed directly by reduced methylviologen. No nicotinamide coenzyme is required. Methylviologen is regenerable electrochemically. Examples of the reduction of enoates, ketones, and 2-oxo acids are given in [155].

2.3 New Alcohol Dehydrogenases from Strains of Rhodococcus and Lactobacillus

None of the commercially available or described alcohol dehydrogenases is able to reduce ketones with bulky side chains such as acetophenone or pinacoline. TBADH when tested with acetophenone [156] was found to be inactive. As optically active phenylethanol and its derivatives are important chiral building blocks, we carried out a limited screening among bacteria and yeasts from a culture collection, in order to isolate NAD(P)-dependent dehydrogenases. This screening resulted in several microorganisms which showed reduction activity for acetophenone or p-Cl-acetophenone in the crude extract [88, 157, 158]. Preliminary results with the most active strains *Rhodococcus erythropolis* DSM 43 297 and *Lactobacillus kefir* DSM 20 587 revealed that these enzymes converted acetophenone and its p-Cl-derivative with an enantiomeric excess of 100%, but yielded in opposite isomers; the enzyme from *Rhodococcus erythropolis* gives the (*S*)-phenylethanols, whereas the *L. kefir* results in the corresponding (*R*)-alcohols [157]. The coenzyme specificity was found to be different, too; the (*S*)-ADH depends on NAD, whereas the (*R*)-ADH accepts NADP only. Both enzymes were purified and characterized and they both proved to be suitable for preparative applications. The characterization of the (*R*)-ADH from *L. kefir* also included microbiological and physiological studies and the reactions with polyclonal antibodies, which led to the detection of a further (*R*)-ADH producer, *Lactobacillus brevis*, which possesses a dehydrogenase with a significantly higher stability.

2.3.1 (*S*)-Alcohol Dehydrogenase from Rhodococcus erythropolis DSM 43 297

S-ADH from *Rhodococcus erythropolis* DSM 43 297 is an important catalyst for the preparation of enantiomerically pure alcohols. It accepts ketones with bulky side chains such as acetophenone, ring-halogenated acetophenones, or pinacoline. An enzyme sample which is already sufficient for technical applications can be obtained by a single chromatographic purification step with an anionic exchanger. The resulting crude enzyme preparation has a specific activity of about 30 U/mg, tested with p-Cl-acetophenone. After four chromatographic purification steps and application of a preparative electrophoresis, the enzyme preparation is pure when tested electrophoretically (Table 4). The specific activity of this sample is 1400 U/mg. Furthermore, the enzyme proved to be quite stable. In a continuous production process, no significant loss of activity could be observed within one week [159]. Some substrates that can be reduced by this enzyme are given in Table 5. (*S*)-ADH from *Rhodococcus erythropolis* converts not only acetophenone and its derivatives and β-ketoesters, but also α-ketoesters (Table 6). Methyl- and ethylpyruvate are rather very good substrates for this enzyme, related to p-Cl-acetophenone they are converted with 13- or 8-fold reaction velocities, respectively. Optically pure α-hydroxyesters are

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Table 4. Purification of (*S*)-ADH from *Rhodococcus erythropolis* DSM 43 297 (1 U = reduction of 1 μmol *p*-Cl-acetophenone per min; HIC = hydrophobic interaction chromatography; Prep-Cell = preparative gel electrophoresis)

Purification step	Total units (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	254	11.5	22	100
Q-Sepharose FF	271	8.3	32.5	100
Phenyl-650 C (HIC)	182	2.35	77	72
Butyl-Sepharose 4FF	142	1.14	124	56
Prep-Cell[a)]	27	0.004	1416	21

[a)] applying 50% of the material from the Butyl-Sepharose chromatography

Table 5. Substrate specificity of the (*S*)-ADH from *Rhodococcus erythropolis* DSM 43 297

Substrate	Concentration [mM]	Relative Activity
Acetophenone	3	27
p-Cl-Acetophenone	3	100
o-Cl-Acetophenone	3	12
m-Cl-Acetophenone	3	294
p-Br-Acetophenone	3	196
p-F-Acetophenone	3	41
p-Methyl-Acetophenone	3	122
p-Methoxy-Acetophenone	3	37
1-Phenyl-propan-2-one	10	12
4-Phenyl-butan-2-one	12	80
6-Methyl-hept-5-en-2-one	12	73

Table 6. Kinetic properties of (*S*)-ADH from *Rhodococcus erythropolis* for the reduction of α-ketoesters

Substrate	Activity at 1 mM substrate conc. (U/mg)	K_m value (mM/L)	V_{max} (%)[a)]
p-Cl-Acetophenone	13.0	—	100
Methylpyruvate	7.8	17.6	1300
Ethylpyruvate	18.6	2.1	823
Ethylbromopyruvate	6.3	—	—
Ethyl-3-methyl-2-oxobutyrate	4.7	—	—
Phenylglyoxalic acid ethylester	3.4	—	—
Phenylglyoxalic acid methylester	3.6	—	—
2-Oxo-4-phenylbutyric acid ethylester	5.0	—	—

[a)] in relation to the v_{max} value (13 U/mg) obtained with *p*-Cl-Acetophenone (measured at pH 6.00)

important chiral building blocks or starting compounds of chiral α-hydroxy acids, which can be released from the esters by cleavage of the ester bond. All hydroxy compounds produced so far have an enantiomeric excess of more than 99%. (*S*)-ADH from *Rhodococcus erythropolis* has a molecular weight of

160 kDa and consists of four identical subunits. With regard to its molecular properties, it belongs to the group of medium-chain ADHs.

2.3.2 (*R*)-Alcohol Dehydrogenase from Lactobacillus Strains

(*R*)-Alcohol dehydrogenase from *L. kefir* was found to reduce acetophenone with a high activity even when used as crude extract. The activity with unpurified cell-free samples was in the range of up to 10 U/mg (reduction of acetophenone). For preparative applications, a technical enzyme sample can simply be reached by only one chromatographic purification step [88]. No desturbing or interfering activities were observed, thus the purification obviously is a suitable means to remove low-molecular weight compounds interfering with the substrate and to standardize and concentrate the enzyme. After chromatography on an anionic exchanger (Mono Q), samples with specific activities of 30 U/mg and more could be obtained (80–90% yield; 5–20-fold enrichment, depending on the starting material). These preparations were suited for first studies about the applicability of the enzyme [88, 160].

2.3.2.1 Purification and Biochemical Characterization of (*R*)-ADH from *Lactobacillus kefir*

A homogeneous enzyme sample was obtained after four chromatographic purification steps (Table 7) including affinity chromatography on 2',5'-AMP-sepharose. In spite of optimization of the purification procedure, severe losses of activity could not be avoided; the yield of the activity of the pure enzyme was in the range of 0.1–3%. The specific activity of the homogeneous protein was in the range of 180 U/mg, when measured with acetophenone as the substrate. The decrease of activity was due to an increasing instability of enzyme in the course of the enrichment. This instability was not a result of the cation dependence of the enzyme. During the first efforts to purify the enzyme it was observed that the activity strictly depends on Mg^{2+} or Mn^{2+}, the addition of 1mM of Mg^{2+} prevents completely the loss of activity.

Table 7. Purification of alcohol dehydrogenase from *Lactobacillus kefir*. (Phenyl- and octylsepharoses are materials for hydrophobic interaction chromatography; Mono Q is an anionic exchanger.)

Purification step	Activity [U/ml]	Specific activity [U/mg]	Yield [%]
Crude extract	32.9	6.3	100
Phenylsepharose	9.7	17.8	23
Octylsepharose	1.7	28.0	10
Mono Q	6.5	97.0	1.7
2',5'-AMP-Sepharose	10.7	149.0	1.0

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Yield [%]
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10
1.7
1.0

The dependence of metal ions is an important feature for the classification of ADHs. Thus, the existence of a weakly bound ion in the *L. kefir* ADH was unexpected and needed assurance by the application of selectively chelating inhibitors. During purification of the crude extract by ion exchangers it was observed, that the activity was almost completely lost. Addition of several cations to such partially inactivated samples resulted in a significant reactivation (130%) by Mg^{2+}-ions. Studies with cation chelating compounds revealed that the ADH from *L. kefir* contains a weakly bound cation. Addition of EDTA deactivates the enzyme completely, whereas chelators for transition metals such as 2,2-dipyridine or 1,10-phenanthroline show no influence on the activity at all. According to the classification of biologically important metals [161], the s-block elements sodium, potassium, magnesium, and calcium interact only weakly with ligands other than oxygen, whereas the transition metals, with partly filled *d*-orbitals, and zinc, bind nitrogen ligands much more strongly. The results with the ADH from *L. kefir* support the assumption that Mg^{2+} is essential for its activity. Purification of the ADH is possible when 1mM of Mg^{2+} is added. In spite of the instability of the ADH from *L. kefir* it was possible to obtain pure enzyme protein.

The preparation of chiral alcohols can be carried out very simply because the regeneration of NADPH is possible by the addition of isopropanol. Unpurified crude extract samples of the ADH from *L. kefir* were found to be a useful catalyst for the synthesis of (*R*)-alcohols [160]; some examples for the preparation of some chiral alcohols using this enzyme are given in Table 8. Though this ADH becomes unstable to such a degree during the purification process, enough material of the pure enzyme could be prepared to produce polyclonal antibodies and to screen for related (*R*)-specific enzymes.

2.3.2.2 Antibody-Supported Screening for (*R*)-Specific Alcohol Dehydrogenases

Several screening procedures among microorganisms were carried out to find enzymes which are capable to reduce acetophenone. The detection of an (*R*)-specific alcohol dehydrogenase in *L. kefir* was the first success of these efforts. Polyclonal antibodies permit to look for the distribution of a given protein among other organisms and to screen for related enzymes. Assays with the commercially available alcohol dehydrogenases from yeast, horse liver and *Thermoanaerobium brockii* indeed, gave no reaction with the antibody; but screening among the genus *Lactobacillus* revealed that each strain of the subgroup *Betabacterium* gave positive results whereas strains of the other subgroups were found to be inactive (Table 9). All the antibody-positive *Betabacterium* strains were assayed with regard to their enzyme activities with acetophenone as the substrate. Only two positive strains were detected, *L. kefir* and *L. brevis*. (Table 10). These results demonstrate that probably each strain of the subgroup of *Betabacterium* possesses a characteristic antibody-positive dehydrogenase, whose biological function is not yet known. The substrate

Table 8. Preparation of chiral alcohols by enzyme-catalyzed reduction of the corresponding ketones with ADH from *Lactobacillus kefir*. The production of phenylethanol with formate and formate dehydrogenase (FDH) for coenzyme regeneration was carried out continuously in an enzyme-membrane-reactor

Alcohol	Method for coenzyme regeneration	Ketone concentration [mM]	Yield [%]	Enantiomeric excess [%]	Ref.
(*S*)-1-Phenyl-2,2,2-trifluoroethanol	isopropanol	150	71	> 99	[160]
(*R*)-1-(2-Pyridyl)-ethanol	isopropanol	150	60	> 97	[160]
(*R*)-1-(2-Furanyl)-ethanol	isopropanol	150	65	95	[160]
(*R*)-6-Methyl-5-hepten-2-ol	isopropanol	150	58	> 99	[160]
(*R*)-5-Chloro-2-pentanol	isopropanol	150	52	> 97	[160]
(*R*)-1-Cyclopropyl-1-ethanol	isopropanol	150	46	> 97	[160]
(*R*)-5-Norbornen-2-ol	sopropanol	150	39	> 97	[160]
(*R*)-1-(Trimethylsilyl)-1-butyn-3-ol	isopropanol	150	25	94	[160]
Methyl-4-hydroxy-1-(trimethylsilyl)-5-hexynoate	isopropanol	150	15	97	[160]
(*R*)-p-Cl-Phenylethanol	isopropanol	10	100	100	[94]
(*R*)-Phenylethanol	isopropanol	5	100	100	[94]
(*R*)-Phenylethanol	formate/FDH	10	> 90	100	[67]
(*R*)-4-Phenyl-2-butanol	formate/rhodium-complex	33	81	96	[162]

Table 9. Taxonomic classification of *Lactobacillus* strains according to Bergey's Manual [163] and their reaction with the *anti-L. kefir*-ADH antibody. *Boldface-typed* strains were tested, *underlined strains* gave a positive reaction with the antibody (*L.* = *Lactobacillus*)

Thermobacterium	Streptobacterium	Betabacterium	
		Group A	Group B
L. acidophilus	***L. casei***	***L. kefir***	***L. hilgardii***
L. helveticus	***L. plantarum***	***L. brevis***	***L. fructivorans***
L. bulgaricus	***L. alimentarius***	***L. cellobiosus***	*L. trichodes*
L. delbrueckii	***L. curvatus***	***L. fermentum***	*L. desidiosus*
L. salivarius	***L. coryneformis***	***L. buchneri***	*L. heterohiochi*
L. leichmanii	***L. farciminis***	***L. viridescens***	
L. lactis	*L. homohiochi*	***L. confusus***	
L. jensenii	*L. xylosus*		

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d]	Enantiomeric excess [%]	Ref.
71	> 99	[160]
50	> 97	[160]
55	95	[160]
58	> 99	[160]
52	> 97	[160]
46	> 97	[160]
39	> 97	[160]
25	94	[160]
15	97	[160]
00	100	[94]
00	100	[94]
90	100	[67]
81	96	[162]

to Bergey's Manual [163] and
strains were tested, *underlined*
is)

um	Group B
	L. hilgardii
	L. fructivorans
us	*L. trichodes*
m	*L. desidiosus*
	L. heterohiochi
ns	

Table 10. Enzyme activity of strains of the betabacterium subgroup A, tested with acetophenone and NADPH

Strain	Activity [U/mg]
L. kefir	87.0
L. brevis	93.0
L. cellobiosus	0.9
L. fermentum	0.2
L. viridescens	0.2
L. confusus	0.3
L. buchneri	0.8

specificity of these dehydrogenases however vary in the various strains of the subgroup, and two of them, *L. kefir* and *L. brevis*, are able to reduce ketones with bulky side chains such as acetophenone. In order to get an impression about the biological function of this dehydrogenase, the physiological characterization of this subgroup is summarized shortly.

The genus *Lactobacillus* was devided by Orla-Jensen [164, 165] into three main subgroups, *Thermobacterium*, *Streptobacterium* and *Betabacterium* basing upon the optimal growth temperatures and fermentation end products. This division has been confirmed by additional physiological tests [166, 167]. Strains of the subgroup *Betabacterium* metabolize glucose in a heterofermentative way to produce lactic acid, CO_2, ethanol and/or acetic acid. The production of CO_2 is a means in practice to distinguish betabacteria from the homofermentative lactobacilli. Further properties of betabacteria are the thiamine requirement for growth, the production of mannitol as an end product of the fructose fermentation and the absence of fructose-1,6-bisphosphate aldolase and triosephosphate isomerase [168, 169]. As these enzymes are lacking, which are essential for the glucose degradation via the fructose-1,6-bisphosphate pathway, glucose is metabolized exclusively by the pentose-phosphate pathway. Subgroup A of the betabacteria consists of fermentatively active species, whereas strains of the subgroup B are inert to most carbohydrates, but grow at pH values as low as 3.2 and on 15% ethanol [163].

2.3.2.3 Induction of the (*R*)-Alcohol Dehydrogenase

Growth studies of *L. kefir* on various carbon sources revealed, that this organism is able to grow likewise on glucose, arabinose, ribose, and lactose, but enzyme activity was found only on glucose and the glucose containing lactose (Table 11). The induction of alcohol dehydrogenase of *L. kefir* by glucose could be demonstrated in the strain by growth on arabinose and different amounts of glucose (Figure 2). Growth in the presence of 0.5% of glucose was found to be optimal for the enzyme activity. In order to obtain information about the kind of activation of the enzyme, crude extracts were prepared by growth on glucose

Table 11. Growth of *Lactobacillus kefir* on various carbon sources (2%) and activity of (*R*)-ADH measured with acetophenone and NADPH. (Optical density was measured at 660 nm)

Carbon source	Growth (Optical density)	Enzyme activity [U/mg]
Glucose	3.4	4.9
Arabinose	3.8	0.7
Ribose	2.0	0
Lactose	3.3	2.1

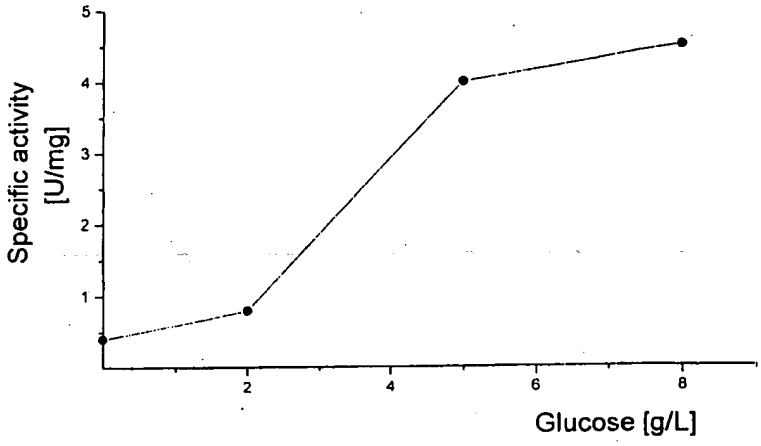

Fig. 2. Induction of (*R*)-alcohol dehydrogenase during growth of *Lactobacillus kefir* on increasing concentrations of glucose

or arabinose, respectively. These crude extracts were used both to demonstrate the enzymatic ability to reduce actophenone and to test the reactivity against polyclonal anti-LKADH antibodies. Within a 3 h incubation, 10 mM of acetophenone were converted completely to (*R*)-phenylethanol with the enzyme extract of cells grown on glucose, whereas no phenylethanol was found within this time with arabinose-grown cells. The antibody-assay was positive with the glucose-grown cells only, no reaction was found with the extract of arabinose-grown cells. Thus it seems evident, that the activation of the alcohol dehydrogenase by glucose occurs at the DNA level.

2.3.2.4 Purification of Alcohol Dehydrogenase from *Lactobacillus brevis*

The ADH from *Lactobacillus brevis* can be purified by exactly the same chromatographic procedures as applied for the purification of *L. kefir* ADH.

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However, the enzyme from *L. brevis* could be eluted from the anionic exchanger at a significantly lower ionic strength, which suggests a different surface charge of both proteins. After application of four chromatographic steps, the enzyme is homogeneous (Table 12). Starting the purification of the *L. brevis* enzyme with the same amount of protein as for the *L. kefir* enzyme, the overall yield was about 10-fold higher due to the higher stability of the *L. brevis* alcohol dehydrogenase.

Enzyme assays with various substrate structures show that a broad range of compounds may be reduced with high activity (Table 13). Thus, the (*R*)-ADHs

Table 12. Purification of alcohol dehydrogenase from *Lactobacillus brevis*. (Phenyl- and octylsepharoses are materials for hydrophobic interaction chromatography; Mono Q is an anionic exchanger.)

Purification step	Activity [U/ml]	Specific activity [U/mg]	Yield [%]
Crude extract	89	30.0	100
Phenylsepharose	57	64.2	51
Octylsepharose	34	86	22.8
Mono Q	37	211	16.6
2', 5'-AMP-Sepharose	64	306	9.6

Table 13. Substrate specificity of alcohol dehydrogenase from *Lactobacillus brevis*

Substrate	Relative Activity [%]
Acetophenone and -derivatives	
Acetophenone	100
4-Cl-Acetophenone	203
3-Cl-Acetophenone	147
2-Cl-Acetophenone	10
4-Ethylacetophenone	65
Propiophenone	17
Benzaldehyde	9
Benzylaceton	99
Methyl-naphthylketone	37
Aliphatic, open-chained ketones	
2,4-Pentandione	130
Hydroxyaceton	23
2-Ketoesters	
Methylpyruvate	98
Ethylpyruvate	248
3-Ketoesters	
3-Ketovaleric acid ethylester	70
3-Ketovaleric acid methylester	69
Cyclic ketones	
2-Methyl-cyclohexanone	192

Table 14. Biochemical properties of the ADHs from *L. kefir* and *L. brevis*

Parameter	*L. kefir*-ADH	*L. brevis*-ADH
Specific activity (acetophenone/NADPH)	760 U/mg	490 U/mg
Coenzyme	NADPH	NADPH
K_m (acetophenone)	0.36 mM	0.85 mM
K_m (NADPH)	0.13 mM	0.16 mM
Molecular mass	105 kDa, homotetramer	105 kDa, homotetramer
Temperature stability (30 min)	37°C	65°C
Temperature optimum	37°C	55°C
pH-optimum (reduction of acetophenone)	7.0	7.0
Dependence on cation	Mg^{2+} (Mn^{2+})	Mg^{2+} (Mn^{2+})

from strains of *Lactobacillus* are useful catalysts for the synthesis of chiral alcohols. The biochemical properties of the (*R*)-ADHs from *Lactobacillus kefir* and *brevis* and data important for their preparative applications are summarized in Table 14.

2.3.2.5 N-Terminal Amino Acid Sequences of (*R*)-Alcohol Dehydrogenases from *L. kefir* and *L. brevis*

The partial N-terminal amino acid sequences obtained by stepwise automated degradation of chromatographically pure ADHs from *L. kefir* and *L. brevis* are summarized in Fig. 3. Both sequences are given at a length of about 45 amino acids of the N-terminus as compared to the sequences of other ADHs. Obviously there is a strong homology of short-chain ADHs in this region to the (*R*)-ADHs from both *Lactobacillus* strains. All related proteins belong to the subgroup of short-chain ADHs.

Within the sequence of the first 40 amino acids of the N-terminus, which is generally regarded as the coenzyme-binding site, six amino acid differ from each other in the *L. brevis* and *L. kefir* ADHs. Three of them are responsible for differing ionic properties of this region, Asn-3 (*L. brevis*) changed into Asp (*L. kefir*), Asp-6 into Lys, and Thr-25 into Asp. The coenzyme-binding sequence G-G-T-L-G-I-G found at the positions 14–20 of *L. brevis* ADH is identical in both enzymes.

2.3.2.6 *L. brevis* ADH Gene and Protein Sequence Determination

After digestion of the homogeneous enzyme with LysC protease and separation of the peptides by HPLC, the sequences of these fragments revealed the C-terminus. The ADH gene was isolated by screening of genomic libraries of *L. brevis* with oligonucleotide probes. The encoding sequence consists of 750 base pairs. Figure 4 summarizes the complete sequence of the *L. brevis* ADH as determined by DNA and peptide sequence analysis. The primary structure consists of 250 amino acid residues.

. *brevis*

L. brevis-ADH
490 U/mg
NADPH
0.85 mM
0.16 mM
ner 105 kDa, homotetramer
65°C
55°C
7.0
Mg^{2+} (Mn^{2+})

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:ohol Dehydrogenases

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brevis) changed into Asp
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revis ADH is identical in

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sequence consists of 750
:e of the *L. brevis* ADH as
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                    + *+ ++ **+  *+* + +    + +** *          +
ADH-Lb          MSNRLDGKVAIITGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEK
ADH-Lk           TDRLKGKVAIVTGGTLGIGLAIADKFVEEGAKVVITGRHADVGEK
ADH-Zm    (41)VPTPMPKRLDGKVAIVTGGARGIGEAIVRLFAKHGARVVIADIDDAAGEA
7HSDH-Ec      MFNSDNLRLDGKCAIITGAGAGIGKEIAITFATAGASVVVSDINADAANH
SDH-Rs          MRLDGKTALITGSARGIGRAFAEAYVREGARVAIADINLEAARE
PGDH-Hs         MHVNGKVALVTGAAQGIGRAFAEALLLKGAKVALVDWNLEAGVQ
AMDG-Fs   TTAGVSRRPGRLAGKAAIVTGAAGGIGRATVEAYLREGASVVAMDLAPRLAAT
BDDDH-Ps        MKLKGEAVLITGGASGLGRALVDRFVAEGAKVAVLDKSAERLAE
KACPR-Ec        MNFEGKIALVTGASRGIGRAIAETLAARGAKVIGTATSENGARI
DCHDDH-Pp      MSDLSGKTIIVTGGGSGIGRATVELLVASGANVPVADINDEAGEA
3BHDH-Ct       TNRLQGKVALVTGGASGVGLEVVKLLLGEGAKVAFSDINEAAGQQ
20BHDH-Sh      MNDLSGKTVIITGGARGLGAEAARQAVAAGARVVLADVLDEEGAA
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Fig. 3. Alignment of the *N*-terminal sequence of the ADH from *Lactobacillus brevis* and homologues proteins.
*, Identity of an amino acid in all proteins; +, identity of an amino acid in six or more proteins. ADH-Lb, alcohol dehydrogenase from *Lactobacillus brevis* [94]; ADH-Lk, alcohol dehydrogenase from *Lactobacillus kefir* [94]; ADH-Zm, alcohol dehydrogenase from *Zea mays* [170]; 7HSDH-Ec, 7α-hydroxysteroid dehydrogenase from *Escherichia coli* [171]; SDH-Rs, sorbitol dehydrogenase from *Rhodobacter sphaeroides* [84]; PGDH-Hs, human 15-hydroxyprostaglandin dehydrogenase [172]; AMDG-Fs, *N*-acylmannosamine-1-dehydrogenase from *Flavobacterium* sp. [173]; DBDDH-Pp, *cis*-1,2-dihydrobenzene-2,2-diol dehydrogenase from *Pseudomonas putida* [174]; BDDDH-Ps, biphenyl-2,3-dihydrodiol dehydrogenase from *Pseudomonas* sp. [175]; KACPR-Ec, ketoacyl-acyl carrier protein reductase homologue from *Escherichia coli* [176]; 3BHDH-Ct, 3β-hydroxysteroid dehydrogenase from *Comomonas testosteroni* [177]; 20BHDH-Sh, 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* [178]

The primary structure of the ADH from *L. brevis* contains several structures which are typical for short-chain ADHs. The N-terminus, with a length of approximately 30 amino acids, is widely regarded as the coenzyme binding site with the conserved motif G-X-X-X-G-X-G, which is G-G-T-L-G-I-G for *Lactobacillus brevis*. A second conserved domain found in the *L. brevis*-ADH sequence is a hydrophobic region comprising 10 or 11 residues, respectively. It contains two highly conserved glycines (G_{82} and G_{92}), separated by nine amino acids. Such structures seem to be located inside the protein and determine the conformation of the enzyme.

A third conserved domain identified in the *L. brevis* ADH begins with a totally conserved tyrosine (Y_{156}). It consists of 18 amino acids ending with a highly conserved acidic amino acid, which is asp in this case. This domain also contains the highly conserved lysine (K_{160}), forming together with tyr-156 a motif previously found in several studies [172, 179, 180]. These amino acid residues are probably involved in the dimerization of enzyme subunits [115] or facilitate the hydride transfer from the alcohol to the cofactor. Crystallographic

ATG TCT AAC CGT TTG GAT GGT AAG GTA GCA ATC ATT ACA GGT GGT ACG
M S N R L D G K V A I I T G G T
TTG GGT ATC GGT TTA GCT ATC GCC ACG AAG TTC GTT GAA GAA GGG
L G I G L A I A T K F V E E G
GCT AAG GTC ATG ATT ACC GGC CGG CAC AGC GAT GTT GGT GAA AAA
A K V M I T G R H S D V G E K
GCA GCT AAG AGT GTC GGC ACT CCT GAT CAG ATT CAA TTT TTC CAA CAT
A A K S V G T P D Q I Q F F Q H
GAT TCT TCC GAT GAA GAC GGC TGG ACG AAA TTA TTC GAT GCA ACG GAA
D S S D E D G W T K L F D A T E
AAA GCC TTT GGC CCA GTT TCT ACA TTA GTT AAT AAC GCT GGG ATC
K A F G P V S T L V N N A G I
GCG GTT AAC AAG AGT GTC GAA GAA ACC ACG ACT GCT GAA TGG CGT
A V N K S V E E T T T A E W R
AAA TTA TTA GCC GTC AAC CTT GAT GGT GTC TTC TTC GGT ACC CGA TTA
K L L A V N L D G V F F G T R L
GGG ATT CAA CGG ATG AAG AAC AAA GGC TTA GGG GCT TCC ATC ATC
G I Q R M K N K G L G A S I I
AAC ATG TCT TCG ATC GAA GGC TTT GTG GGT GAT CCT AGC TTA GGG GCT
N M S S I E G F V G D P S L G A
TAC AAC GCA TCT AAA GGG GCC GTA CGG ATT ATG TCC AAG TCA GCT
Y N A S K G A V R I M S K S A
GCC TTA GAT TGT GCC CTA AAG GAC TAC GAT GTT CGG GTA AAC ACT GTT
A L D C A L K D Y D V R V N T V
CAC CCT GGC TAC ATC AAG ACA CCA TTG GTT GAT GAC CTA CCA GGG
H P G Y I K T P L V D D L P G
GCC GAA GAA GCG ATG TCA CAA CGG ACC AAG ACG CCA ATG GGC CAT
A E E A M S Q R T K T P M G H
ATC GCT GAA CCT AAC GAT ATT GCC TAC ATC TGT GTT TAC TTG GCT TCT
I G E P N D I A Y I C V Y L A S
AAC GAA TCT AAA TTT GCA ACG GGT TCT GAA TTC GTA GTT GAC GGT GGC
N E S K F A T G S E F V V D G G
TAC ACT GCT CAA
Y T A Q

Fig. 4. DNA and protein sequence of the recombinant (*R*)-alcohol dehydrogenase from *Lactobacillus brevis* in *E. coli* (*upper line*: DNA sequence; *lower line*: corresponding amino acid in one letter code). The sequences of the primers are given in *bold-type*, sequences obtained by amino acid sequencing are *underlined*

studies on 3α,20β-hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* revealed that the conserved tyrosine is located near the pyridine ring of the coenzyme and the conserved lysine is directly behind the tyrosine. Chemical modification and site-directed mutagenesis of short-chain ADHs demonstrate

GGT GGT ACG
G G T
A GGG
E G
AA AAA
E K
C CAA CAT
F Q H
A ACG GAA
A T E
G ATC
G I
GG CGT
W R
C CGA TTA
R L
TC ATC
I I
A GGG GCT
L G A
A GCT
S A
C ACT GTT
N T V
A GGG
P G
GC CAT
G H
GCT TCT
L A S
AC GGT GGC
D G G

ol dehydrogenase from *Lacto-*
onding amino acid in one letter
ences obtained by amino acid

treptomyces hydrogenans
the pyridine ring of the
d the tyrosine. Chemical
hain ADHs demonstrate

the involvement of this strictly conserved tyrosine [181–184]. From photoaffinity labelling studies on estradiol 17β-dehydrogenase it can be assumed that the C-terminal part of the protein is responsible for the substrate specificity of short-chain dehydrogenases [185].

The N-terminal sequences and the molecular mass of the subunits of the (*R*)-ADH from *L. kefir* and *L. brevis* clearly demonstrate that these new ADHs belong to the subgroup of short-chain dehydrogenases. Within this group these new dehydrogenases are the first examples of metal containing and metal requiring enzymes. Further studies concerning the structure-function relationship of these parts of the enzyme are necessary to elucidate the role of the cations.

3 Alcohol Dehydrogenases as Technical Catalysts

The biochemical characterization of several alcohol dehydrogenases and their exploitation potential demonstrate that these enzymes are most important tools for biochemists. Amino acid sequences of several ADHs are available so far, and alignment studies allow to establish ADH families and to consider their probable evolutionary relationships. For preparative applications, however, particular properties of an enzyme are essential prerequisites, such as enzyme stability and availability, its substrate specificity, or reaction selectivity. Enzymes with NAD as coenzyme are clearly preferred to NADP-dependent ones in practice, because NAD has a significantly higher stability [186–188], a lower price and, is in general, easier to regenerate.

3.1 Whole Cell vs. Enzymes as Means for the Reduction of Ketones

Alcohol dehydrogenases can be applied for the synthesis of chiral hydroxy compounds, either as whole cells or as a cellfree, more or less purified enzyme extract. As discussed in the introduction, whole cells are easy to use; coenzyme regeneration can simply be performed by the metabolic pathways of the cells, which can be fed by adding a degradable carbon source. Furthermore, the expensive isolation of the alcohol dehydrogenase can be avoided. However, there are several disadvantages: cells frequently contain enzymes that interfere with the desired reaction, so that a low yield and low enantiomeric excess of the product can be the result. Nevertheless, transformations with bakers' yeast have found their place in organic syntheses. This catalyst can be applied almost without any microbiological or biochemical experiences and is readily available. Remarkably, all these applications are limited to reduction reactions, whereas isolated enzymes in fact can be used in both directions, demanding separate coenzyme regeneration methods, only. Oxidation of alcohols requires particular

strains of microorganisms, such as *Xanthobacter autotrophicus* to oxidize 2-chloroethanol [189], *Pseudomonas* sp., which oxidizes alkan-2-ols [190]; or methylotrophs [191–196].

In general, syntheses with isolated enzymes can be performed with higher selectivity and space-time yield than with whole cells, but they require in any case the coupling of coenzyme regenerating reactions.

3.2 Regeneration of Nicotinamide Coenzymes

Generally, the nicotinamide coenzymes are not covalently bound to the enzyme. They are employed in enzyme assays and preparative applications by adding catalytical but optimized amounts, and they need to be recycled. For an economic process, an efficient regeneration method is a basic requirement. The necessary recycle number depends essentially on the value of the chiral product, generally the method should recycle the coenzyme 100–100,000 times ([42]).

Numerous systems have been developed so far for the regeneration of the reduced coenzymes [197]. One of these is a chemical method with dithionite [198, 199], which is inexpensive, but unstable and which may reductively inactivate enzymes. Photochemical methods for coenzyme regeneration require photosensitizer such as tris(bipyridine)ruthenium(II) [200, 201] or *meso*-tetramethylpyridinium-porphyrinzinc(II) [202]. Electrochemical regeneration requires a suitable mediator, because direct cathodic reduction of NAD(P) generates undesirable byproducts of the coenzymes. Methylviologen has been widely used for mediator, but the reduced form requires a reductase for the reduction of NAD(P). Thus, this method is limited to the use of whole cells (electromicrobial reductions), that usually contain methylviologen-dependent NAD(P) reductases [154, 155, 203–205]. A non-enzymatic regeneration system for NADH and NADPH with a rhodium-bipyridinium complex as the redox catalyst and either the electrode or formate as the donor was developed recently by Steckhan et al. [206–208]. The formate-driven regeneration of NADH and NADPH was demonstrated for the stereoselective reduction of 4-phenyl-2-butanone with several alcohol dehydrogenases (HLADH, TBADH, (*S*)-ADH from *Rhodococcus erythropolis* and (*R*)-ADH from *Lactobacillus kefir*) [162]. Each system results in 4-phenyl-2-butanol with an enantiomeric excess of more than 96%. A water-soluble high-molecular weight derivative of this rhodium complex [209] facilitates continuous reduction reactions.

The most convenient and useful enzymatic methods for the regeneration of NAD(P)H are formate/formate dehydrogenase for NADH [210, 211], isopropanol/TBADH for NADPH [57], isopropanol/ADH (*Pseudomonas* sp.) for NADH [61, 212] and glucose/glucose dehydrogenase (*Bacillus* sp.) for NADH and NADPH [213].

For the regeneration of NADH, formate and formate dehydrogenase are most widely used. The enzyme from *Candida boidinii* is commercially available, formate is inexpensive, and the equilibrium favours a nearly irreversible

reaction producing CO_2, and this product can easily be separated from the product solution. Disadvantages are the low specific activity of formate dehydrogenase (3–4 U/mg) and its price. This enzyme was already applied in large-scale processes using a continuously working enzyme-membrane-reactor. For this device, NAD was used as a molecular-weight enlarged derivative, which was obtained by chemical covalent binding of NAD to high-molecular weight polyethyleneglycole. The applicability of this technology was demonstrated by several applications in the field of enantioselective synthesis of hydroxy and amino acids (for a review see [2, 3]). Up to 600,000 moles of product may be produced per mole of NAD in such processes. Further limitation of the commercially available formate dehydrogenase arises from the fact that this enzyme exclusively reacts with NAD. Quite recently however, a formate dehydrogenase has been developed, which accepts both NAD and NADP [68, 69]. It could be achieved to broaden the coenzyme specificity by multipoint site-directed mutagenesis of the gene encoding for the NAD-dependent formate dehydrogenase in *Pseudomonas* sp. [214]. The activity of the mutant enzyme with NADP is in the range of 60% as compared to the NAD reduction. This enzymatic system may be applied in a continuous enzymatic reduction process to reduce acetophenone with the NADP-dependent ADH from *Lactobacillus* sp. [67].

The regeneration of the oxidized coenzymes NAD(P) is required for the synthesis of ketones from the racemic mixture of the corresponding hydroxy compounds. Besides the synthesis of enantiomerically pure hydroxyketones as the oxidation product of diols, this kind of reaction is important if ketones as the starting material are unstable or difficult to prepare. As a prerequisite to produce enantiomerically pure hydroxy compounds in this way the oxidation reaction must proceed completely. Methods for the regeneration of the oxidized nicotinamide coenzymes are less well developed than for the reduced coenzymes. Basically, regeneration of the oxidized coenzymes is hampered by the unfavourable thermodynamics and also by product inhibition [141]. Nevertheless, there are enzymatic methods for the regeneration based on α-ketoglutarate/L-glutamate dehydrogenase [141, 215–218], pyruvate or glyoxylate/lactate dehydrogenase [219] or acetaldehyde/ethanol dehydrogenase [220, 221]. A simple non-enzymatic method with flavin mononucleotide (FMN) was developed by Jones and Taylor [222–224]. FMN oxidizes NADH and FMN is afterwards regenerated by O_2.

In fact, the α-ketoglutarate/glutamate dehydrogenase is a generally applicable method for the regeneration of NAD and NADP in laboratory scale productions. Both components involved are inexpensive and stable. Quite recently, a method for the oxidation of the reduced nicotinamide coenzymes based on bacterial NAD(P)H oxidase has been described [225]. This enzyme oxidizes NADH as well as NADPH with low K_m values. The product of this reaction is peroxide, which tends to deactivate enzymes, but it can be destroyed simultaneously by addition of catalase. The irreversible peroxide/catalase reaction favours the ADH catalyzed oxidation reaction, and complete conversions of this reaction type are described.

3.3 Dehydrogenases in Organic Solvents

Substrates of alcohol dehydrogenases very often are almost insoluble in water. On the other hand, enzymes are generally isolated in aqueous buffers, and catalysis performed in aqueous solutions. Although enzymes as a rule are destroyed by the addition of organic especially water-miscible solvents, it has become evident in the last years, that enzymes may be active in the presence of organic solvents [226–230]. This was confirmed for enzymes that work originally in a hydrophobic environment, for instance in a biological membrane, as well as for very stable enzymes, such as proteases or enzymes from thermophilic organisms. The stability of enzymes in organic solvents depends on the hydrophobicity of the solvent, which can be expressed as the log P-value [231, 232]. Enzymes are more stable in nonpolar solvents with a low solubility for water than in polar solvents. In general, enzymes need a however small amount of water to retain a thin water layer on the surface, which is necessary to maintain the catalytically active conformation [233–237]. Coenzyme-dependent enzymes are rarely used in organic solvents. They are even less stable and less active in organic solvents than enzymes which require no cofactors; moreover, the coenzyme regeneration step certainly is an additional complication for such applications. Being a prerequisite for the application of alcohol dehydrogenases in organic solvents, their activity and stability were tested in the presence of nonpolar solvents (Table 15). These studies indicate that ADHs remain stable in the presence of organic solvents with a log P value of 3.5 or higher, solely YADH is unstable in all organic solvents tested so far. However, no results of preparative applications of ADHs in organic solvents are available until now.

Table 15. Influence of organic solvents on activity and stability of alcohol dehydrogenases. Values for the stability are given as the residual activity after 5-h contact with the solvents. The solvents are characterized and classified according to their log P-value [231]. Enzymes are active or stable in the presence of solvents at the mentioned log P value or higher. (Solvents in brackets are those that correspond to the mentioned log P-value.)

Alcohol dehydrogenase	Influence of Organic Solvents on	
	Stability	Activity
Lactobacillus kefir	log P > 2.5 (Toluol)	log P > 2.5 (Toluol)
Rhodococcus erythropolis	log P > 4 (Heptane)	log P > 3.5 (Hexane)
HLADH	log P > 1.2 (t-Butylmethylether)	log P > 1.2 (t-Butylmethylether)
TBADH	log P > 1.5 (Diisopropylether)	log P > 3 (Trichlorethan)
YADH	all solvents deactivate the enzyme completely (tested up to log P = 6.6 (Dodecan))	

: almost insoluble in water.
:d in aqueous buffers, and
gh enzymes as a rule are
ter-miscible solvents, it has
be active in the presence of
enzymes that work origin-
a biological membrane, as
enzymes from thermophilic
ents depends on the hydro-
the log P-value [231, 232].
ı a low solubility for water
ı however small amount of
ich is necessary to maintain
enzyme-dependent enzymes
ess stable and less active in
factors; moreover, the coen-
mplication for such applica-
alcohol dehydrogenases in
: tested in the presence of
that ADHs remain stable in
f 3.5 or higher, solely YADH
vever, no results of prepara-
available until now.

of alcohol dehydrogenases. Values
with the solvents. The solvents are
Enzymes are active or stable in the
olvents in brackets are those that

vents on	
	Activity
	log P > 2.5 (Toluol)
	log P > 3.5 (Hexane)
	log P > 1.2 (t-Butylmethylether)
	log P > 3 (Trichlorethan)
e enzyme completely .6 (Dodecan))	

Table 16. Chiral alcohols produced by continuous enzyme-catalyzed processes. The corresponding ketones are reduced with (*S*)-ADH from *Rhodococcus erythropolis*, NADH was regenerated by simultaneous coupling with formate dehydrogenase from *Candida boidinii* (FDH) and formate (data from [159])

Product alcohol	Ketone concentration [mM]	Space-time yield [$g \cdot L^{-1} \cdot d^{-1}$]	Enzyme consumption [$U \cdot kg\ (Product)^{-1}$]	
			(*S*)-ADH	FDH
(*S*)-1-Phenylpropan-2-ol	9.0	64	3540	10200
(*S*)-4-Phenylbutan-2-ol	12.0	104	3025	4860
(*S*)-6-Methylhept-5-en-2-ol	10.0	60	n.d.	n.d.

A more promising approach for the synthesis of hydrophobic substances with ADHs is published by Kruse et al. [159, 238]. They use a continuously operating reactor where the enzyme containing water phase is separated from the hydrophobic substrate-containing organic phase by a membrane. The hydrophobic product is extracted continuously via a hydrophobic membrane into an hexane phase, whereas the coenzyme is regenerated in a separate cycle, that consists of a hydrophilic buffer system. This method decouples advantageously the residence time of the cofactor from the residence time of the substrate. Several hydrophobic alcohols were prepared in this way with (*S*)-ADH from *Rhodococcus erythropolis* (Table 16).

3.4 Dehydrogenase-Catalyzed Preparation of Chiral Alcohols

Methods to produce chiral alcohols with ADHs are essentially described at a laboratory scale, namely those using HLADH, TBADH and the recently isolated enzymes from *Rhodococcus erythropolis*, and *Lactobacillus kefir* and *L. brevis*, respectively.

HLADH was applied for the reduction of a broad variety of substrates. The regeneration of NADH can be achieved by the ethanol coupled-substrate method [239]. Cyclic ketones are good substrates for this enzyme [137, 240, 241]. Heterocyclic compounds containing oxygen or sulfur are accepted, too [242–246], but nitrogen-containing substrates are inhibitors of HLADH, because they apparently complex with zinc in the active site of the enzyme [247].

Enantioselective reduction reactions with HLADH were carried out for instance with 2- and 3-keto esters [213, 248], *cis* and *trans* decaindiones [121] or cage shaped molecules [249].

As for oxidation reactions catalyzed by HLADH, the most frequently reported method is the coupling with FMN [222–224]. It has been used for instance for the oxidation of many meso-diols forming lactones [250, 251], or for the oxidation of primary alcohols to obtain chiral aldehydes [252]. Generally, these syntheses were carried out at 1–2 g scale within a reaction time of a few hours up to 2–3 weeks [247].

The NADP-dependent TBADH was used for the laboratory-scale preparation of several chiral aliphatic and cyclic hydroxy compounds by reduction of the corresponding ketones. For the regeneration of NADPH, this reduction reaction can be coupled with the TBADH catalyzed oxidation of isopropanol. For the reduction of some ketones it was observed that the reaction rate was increased in the presence of the regenerating substrate isopropanol, for instance in the presence of 0.2 v/v isopropanol, the reduction rate of butanone or pentanone was increased 3–4-fold [57]. In some cases, the enantiomeric excess of the reduction reaction is not very high, especially when small molecules are converted, but also for compounds such as acetophenone [138].

(*R*)-alcohols in high enantiomeric excess can be obtained with the aid of the NADP-dependent ADH from *Lactobacillus kefir*. Due to the broad substrate specificity of this enzyme, aromatic, cyclic, polycyclic as well as aliphatic ketones can be reduced. A simple method for the regeneration of NADPH is given by the simultaneously coupled oxidation of isopropanol by the same enzyme. Several chiral alcohols (Table 8) were synthesized at a 2.5 mmol scale within a reaction time of 12–36 h [160].

The preparation of some (*S*)-alcohols by ADH from *Rhodococcus erythropolis* has been described quite recently. This was the first report of a continuous production process for hydrophobic compounds. An important prerequisite of this method is a membrane which is resistant to organic solvents. It separates the hydrophilic phase, which contains the enzyme and the coenzyme, from the hydrophobic phase with the substrate and the product. Several products were prepared with this enzyme at a multigram scale (Table 16).

In summary, there are several alcohol dehydrogenases available for the synthesis of chiral alcohols. Most of the described enzymes belong to one of three groups which are precisely characterized. For preparative applications, only a few of these enzymes can be used, for their availability, stability, or substrate specificity may be insufficient. Only HLADH, TBADH, (*R*)-ADH from *Lactobacillus kefir* or *L. brevis* and the (*S*)-ADH from *Rhodococcus erythropolis* were found to be enzymes for general use. Until now, preparations of chiral alcohols have been carried out in laboratory-scale only; no product has been produced so far in a kilogram- or ton-scale by alcohol dehydrogenases. The low productivity of some of these enzymes surely means a limitation to the applicability. For instance, HLADH catalyzes the conversion of 1 mmol alcohol/day with about 20 mg of enzyme. Another drawback are the economics of this enantioselective step. For the conversion of water-soluble substrates such as keto acids, the product related enzyme consumptions are in the range of 500–2000 U/kg of product (Table 16). Hydrophobic substances, however, require significantly higher amounts of enzyme, which are in the range of 3000 U/kg for the ADH and 5000–10,000 U/kg for the formate dehydrogenase mediated regeneration step [159]. Enzyme costs of 500–1000 $/1000 U for some ADHs and similar costs for formate dehydrogenase must hence be compared to alternate non-biological processes for the production of chiral alcohols.

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Appendix C

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Pichia stipitis Genes for Alcohol Dehydrogenase with Fermentative and Respiratory Functions

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Two genes coding for isozymes of alcohol dehydrogenase (ADH), designated *PsADH1* and *PsADH2*, have been identified and isolated from *Pichia stipitis* CBS 6054 genomic DNA by Southern hybridization to *Saccharomyces cerevisiae ADH* genes, and their physiological roles have been characterized through disruption. The amino acid sequences of the *PsADH1* and *PsADH2* isozymes are 80.5% identical to one another and are 71.9 and 74.7% identical to the *S. cerevisiae* ADH1 protein. They also show a high level identity with the group I ADH proteins from *Kluyveromyces lactis*. The PsADH isozymes are presumably localized in the cytoplasm, as they do not possess the amino-terminal extension of mitochondrion-targeted ADHs. Gene disruption studies suggest that PsADH1 plays a major role in xylose fermentation because *PsADH1* disruption results in a lower growth rate and profoundly greater accumulation of xylitol. Disruption of *PsADH2* does not significantly affect ethanol production or aerobic growth on ethanol as long as *PsADH1 is* present. The PsADH1 and PsADH2 isozymes appear to be equivalent in the ability to convert ethanol to acetaldehyde, and either is sufficient to allow cell growth on ethanol. However, disruption of both genes blocks growth on ethanol. *P. stipitis* strains disrupted in either *PsADH1* or *PsADH2* still accumulate ethanol, although in different amounts, when grown on xylose under oxygen-limited conditions. The *PsADH* double disruptant, which is unable to grow on ethanol, still produces ethanol from xylose at about 13% of the rate seen in the parental strain. Thus, deletion of both *PsADH1* and *PsADH2* blocks ethanol respiration but not production, implying a separate path for fermentation.

Reduction of acetaldehyde to ethanol is the last step in ethanol production. Conversely, ethanol oxidation is the first reaction of ethanol respiration. The enzymes catalyzing these reactions and their structural genes have been well studied in *Saccharomyces cerevisiae*. Alcohol dehydrogenase (EC 1.1.1.1), ADH1, the classical fermentative isozyme, is responsible for the last step in the yeast glycolytic pathway, the reduction of acetaldehyde to ethanol (4, 8, 11). ADH2, the oxidative isozyme, is highly repressed by fermentative conditions (12) and is derepressed in the absence of a fermentable sugar such as glucose. The function of ADH2 in the cell is to oxidize ethanol, formed during fermentation, to acetaldehyde, which can then be metabolized via the tricarboxylic acid cycle in the mitochondria and also serve as an intermediate in gluconeogenesis (30). *S. cerevisiae* has two other *ADH* genes: *ADH3*, coding for a mitochondrion-targeted enzyme presumably involved in ethanol oxidation (48), and *ADH4*, which displays no significant similarity to any other characterized yeast *ADH* gene (46). Four such ADHs have been characterized in the galactose-fermenting yeast *Kluyveromyces lactis* (23, 32, 33, 38). These genes, named *KlADH1* through *KlADH4*, encode two cytoplasmic (KlADH1 and KlADH2) and two mitochondrial (KlADH3 and KlADH4) activities. *KlADH1* and *KlADH2* are preferentially expressed in the presence of glucose (33, 38). By contrast, *KlADH4* is induced at the transcriptional level in the presence of ethanol (23, 38). Three of the ADH isozymes from *S. cerevisiae* and the four isozymes of *K. lactis* all belong to the group I zinc-dependent enzymes because of their sequence identity and functional similarities (27). The ADH enzymes of xylose-fermenting yeasts have not been examined previously.

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The xylose-fermenting yeast *Pichia stipitis* is among the few organisms that use both xylose and glucose and exhibit a regulatory transition between respiratory and fermentative processes (19). The fundamental mechanisms by which fermentation is regulated differ profoundly in the glucose-fermenting yeast *S. cerevisiae* and the xylose-fermenting yeast *P. stipitis* (16 19, 45). In *S. cerevisiae*, the availability of oxygen is irrelevant to fermentative metabolism. Glucose induces high levels of glycolytic enzymes and represses respiration, leading to ethanol production (4). In the Crabtree-negative yeast *P. stipitis*, oxygen limitation, rather than the presence of either glucose or xylose, induces fermentation (25, 45).

P. stipitis is capable of producing ethanol from xylose under anaerobic conditions because it has a single xylose reductase with dual cofactor (NADPH and NADH) specificity (1). In most yeasts and fungi, xylose reductase uses only NADPH. Xylose metabolism in yeasts proceeds via xylose reductase (XR), which catalyzes the reduction of xylose with NAD(P)H to form xylitol, and xylitol dehydrogenase (XDH), which catalyzes the oxidation of xylitol by NAD to form xylulose. Thus, a cofactor imbalance can arise under anaerobic conditions if XR uses only NADPH, XDH uses only NAD, and no mechanism exists to reduce NADP with NADH. In *P. stipitis*, XR can accept either NADPH or NADH, so a cofactor imbalance does not block xylose uptake under anaerobic conditions (7).

P. stipitis requires small amounts of oxygen for maximal conversion of Xylose to ethanol (41). Under strictly anaerobic conditions, little ethanol is formed (19), The explanation for this oxygen effect could reside in the higher activity of its X with NADPH. Its XDH is specific for NAD, so partial accumulation of NADP and NADH probably still occurs, despite NADH-linked XR activity in *P. stipitis* (20).

Two key fermentative enzyme activities, pyruvate decarboxylase (PDC) and ADH, are induced as *P. stipitis* becomes oxygen limited and ethanol production increases (25). How-

TABLE 1. *P. stipitis* strains used in this study

Strain	Genotype	Origin	Reference
CBS 6054 (= NRRL Y-11545 = ATCC 58785)	Wild type	CBS[a]	20
FPL-PSU-1	*Psura3-2/Psura3-2*	CBS 6054	47
FPL-PLU20	*Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1*	FPL-DX26 (= NRRL Y-21304)	22
FPL-PSU218	*Psura3-2/Psura3-2 Psadh1::PsURA3/Psadh1::PsURA3*	FPL-PSU1 (= NRRL Y-21446)	This study
FPL-PLU123	*Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1 Psadh2::PsLEU2/Psadh2::PsLEU2*	FPL-PLU20	This study
FPL-PLU1209	*Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1 Psadh1::PsURA3/Psadh1::PsURA3 Psadh2::PsLEU2/Psadh2::PsLEU2*	FPL-PLU123	This study

[a] CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

ever, little is known about this process in *P. stipitis*, at the molecular genetic and physiological levels. A better understanding of metabolic regulation of the genes involved in xylose fermentative metabolism is essential to further advance this field. A recent paper from our laboratory described two *P. stipitis* genes for PDC (21). The next step toward understanding the roles of fermentation and respiration in xylose metabolism is to clone and disrupt *P. stipitis ADH* genes.

The objective of the present study was to isolate and characterize the *ADH* genes necessary for ethanol production in *P. stipitis*. The research identified two distinct genes for ADH in *P. stipitis*. We determined the physiological roles for each and showed that one *(PsADH1)* is critically important for ethanol production.

MATERIALS AND METHODS

Microbial strains. The *P. stipitis* strains used in this research are summarized in Table 1. *P. stipitis* CBS 6054 was the source of all derived strains and of all sequenced DNA. *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.) (F *recA1 endA1 hsdR17* [$r_k^- m_k^+$] *supE44 thi-1 gyrA relA1*) was used for all recombinant DNA experiments that required a bacterial host.

Media and culture conditions. Yeasts were grown in yeast-peptone-dextrose medium consisting of yeast extract at 10 g/liter, peptone at 20 g/liter, and glucose at 20 g/liter. For cultivation of *ura3* and *leu2* auxotrophs, media were supplemented with 100 mg of uridine per liter and 100 mg of leucine per liter, respectively. *E. coli* was grown in Luria-Bertani medium (34) with 50 μg of ampicillin per ml in liquid medium or 100 μg of ampicillin per ml in solid medium. Fermentation studies were done with 1.7 g of yeast nitrogen base per liter without ammonium sulfate or amino acids (YNB; Difco, Detroit, Mich.) supplemented with Bacto Peptone at 6.56 g/liter, urea at 2.27 g/liter (2 × nitrogen), and 80 g of D-xylose per liter plus leucine or uridine as needed. Cells were cultivated at 25°C in 50 ml of fermentation medium shaken in 125-ml Erlenmeyer flasks at 200 rpm.

Plasmid constructions. The *PsURA3* (47) and *PsLEU2* (22) selectable markers originated from *P. stipitis* CBS 6054. Plasmid Bluescript KSII ⁺was obtained from Stratagene (La Jolla, Calif.). pJY102 *(PsADH1* disruption cassette) was built in three steps (i) the *EcoRI* site in pUC19 was destroyed by treatment with T4 DNA polymerase (New England Biolabs, Beverly, Mass.) and ligation reactions to form pJY19; (ii) a 2.0-kbp *Sac I-Sac* I fragment of the *PsADH1* gene bearing a unique *EcoRI* site was inserted into the *Sac* I site of pJY19 to form PJY101; and (iii) a plasmid containing the *URA3* gene of *P. stipitis*, pVY3 (47), was cut with *Pst* I (New England Biolabs). The *Eco* RI linker (New England Biolabs) was inserted at the remaining *Pst* I site. A 1.2-kbp *Eco* RI - *Eco* RI fragment carrying the *P. stipitis URA3* gene was inserted into a unique *Eco* RI site within the *PsADH1* gene of pJY101 to form PJY102. The resulting plasmid, pJY102, contained the 3.2-kbp *Sac* I- *Sac* I *Psadh1::PsURA3* fragment and was digested with *Sac* I prior to transformation. PJY202 *(PsADH2* disruption cassette) was built in three steps: (i) a portion (3.5 kbp) of the 5'-flanking region was deleted from *PsADH2* to leave a unique *Pst* I site in its coding region; (ii) this site was destroyed by T4 DNA polymerase, and a 2.1-kbp blunt-ended *Eco* RI fragment bearing *PsLEU2* gene was ligated into the middle of *Psadh2* to form pJY201, and (iii) a 4.5-kbp *Sac* I -*Bam* HI fragment of pJY201 bearing the *Psadh2::PsLEU2* construct was inserted into the corresponding sites of pUC18 to form pJY202. The resulting plasmid, pJY202, was digested with *Sac* I and *Bam* HI prior to transformation.

Yeast transformation. Lithium acetate transformation of *P. stipitis* PSU1 *(ura3-2/ura3-2)* and PLU20 *(ura3-3/ura3-3 leu2 Δ-1/leu2 Δ-1)* (22) was performed as described by Ito et al. (18). Yeast transformants were selected on YNB plus 20 g of glucose per liter without uracil or leucine when *URA3* and *LEU2* were used as selectable markers, respectively. For solid media, 20 g of agar per liter was added.

DNA isolation. Plasmid DNA was isolated and purified by the alkaline extraction method of Birnboim and Doly (5) or with the Qiagen Plasmid Prep kit (Qiagen Corp., Chatsworth, Calif.). Yeast genomic DNA was isolated and purified as described by Specht et al. (43) or Rose et al. (28).

Cloning of *P. stipitis ADH* genes. Genomic DNA from *P. stipitis* CBS 6054 was cut with *Bam* HI and *Sal* I and electrophoresed in 0.8% agarose. DNA corresponding to ca. 6.6 kbp was isolated from the gel slice by using Gelase (Epicentre Corp., Middleton, Wis.). The purified DNA fragment was ligated into the corresponding sites of pBluescript KSII. The DNA library was amplified in *E. coli* DH5α, followed by electroporation using the Pulse Controller together with the Gene Pulser from Bio-Rad (Richmond, Calif.). Inserts containing *ADH* sequences were identified by colony hybridization to the PCR-amplified *S. cerevisiae ADH1* coding sequences, and colonies that were positive with the probe were tested further. The second genomic library was constructed from *Bam* HI- and *Xba* I-digested CBS 6054 genomic DNA as described above for the isolation of *P. stipitis ADH1*. *E. coli* transformants were screened for the presence of *S. cerevisiae ADH2* homologous sequences by using the colony hybridization technique (34).

Blot hybridizations. Genomic DNA digests were electrophoresed in 0.8% agarose, blotted into Nytran filters (Schleicher & Schuell, Keene, N.H.) by standard procedures (42), and hybridized with the PCR-amplified *P. stipitis PsADH2* or *S. cerevisiae ADH1* or *ADH2* coding sequences labeled with digoxigenin-11-dUTP by using a Genius 1 kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hybridizations were done at 37°C under the manufacturer's (Boehringer Mannheim) specified conditions. The colony hybridizations were performed under standard conditions (34) by using a Genius 1 kit (Boehringer Mannheim).

DNA sequencing. The dideoxy method of Sanger et al. (35) was used for DNA sequencing (Sequenase version 2.0 DNA sequencing kit; United States Biochemical, Cleveland, Ohio). Primers were synthesized at the University of Wisconsin–Madison Biotechnology Center and Ransom Hill Bioscience, Inc. (Ramona, Calif.). Nested deletions of pJY158, a plasmid containing the *PsADH2* gene, were sequenced from universal primers located adjacent to the multiple cloning site. The sequence was confirmed by sequencing other deletions with overlapping endpoints. The sequence of the coding region and 5'- and 3'-flanking regions is available under GenBank accession no. AF008244. The *Spe* I- *Xba* I fragment of pJY268, a plasmid containing *PsADH1* (see Fig. 2A), was deleted, religated, and subjected to sequence analysis by primer walking from the universal primers located adjacent to the multiple cloning site. The sequence of the coding region and the 5'- and 3'-flanking regions is available under GenBank accession no. AF008245. The Genetics Computer Group programs (13) developed at the University of Wisconsin-Madison were used to evaluate DNA and derived amino acid sequences.

PCR screening of *PsADH* disruptants. Amplification reactions were performed in 50 μl containing 100 ng of genomic template DNA, 200 pmol of each primer, 0.2 mM each deoxyribonucleoside triphosphate (A, T, C, and G), and 5 U of *Pfu* DNA polymerse (Stratagene) in 10 mM KCl–10 mM $(NH_4)_2SO_4$–20 mM Tris-Cl (pH 8.8)–2 mM $MgSO_4$–0.1% Triton X-100–100-μg/ml bovine serum albumin (the 10× reaction buffer provided with the enzyme). Times and temperatures were as follows: 5 min at 94°C, 1 min at the primer annealing temperature, and 6 min at 75°C. The reaction was completed by a 10-min incubation at 75°C. Amplification was performed in a Coy Temp Cycler II (Coy Corp., Grass Lake, Mich.). The PCR products were checked by gel electrophoresis. Primers, supplied by Genosys Biotechnologies, Inc. (The Woodlands, Tex.), were as follows (sequences are 5' to 3'); no. 50 (20-mer near the 5' end of the *LEU2* insertion at *PsADH2*), TGT-CTG-TCA-CAC-CGA-CTT-GC; no. 34(20-mer near the 3' end of the *LEU2* insertion at *PsADH2*), TGG-CTT-CAG-AGT-CAG-CT; no. 53 (18-mer near the 5' and of the *URA3* insertion at *PsADH1*),

GCT-CTA-CAA-GGA-CAT-TCC: no. 35 (18-mer near the 3' end of the *URA3* insert at *PsADH1*). CCT-GGT-TGG-ATT-TGA-GCG.

Analytical methods. Determination of ethanol, xylose, and xylitol was done as described previously (9). Cell growth was determined daily by monitoring optical density at 600 nm (OD_{600}), and cell dry weight was estimated by diluting a suspension to OD_{600}s between 0.15 and 0.5. Under these conditions, an OD_{600} of 1.0 equals 0.24 g of cells/liter.

RESULTS

Identification of *PsADH* genes. *P. stipitis* genomic DNA was digested with *Bam* HI and *Sal* I, and the fragments containing *ADH* genes were identified by Southern hybridization using *S. cerevisiae ADH1 (ScADH1)* as a probe. *S. cerevisiae* genomic DNA was used as a control. The *P. stipitis* genome showed two bands hybridizing to *ScADH1*. As expected, the *S. cerevisiae* genome showed at least three restriction fragments hybridizing to the probe.

Cloning of *PsADH2*. One fragment of *P. stipitis* DNA, 6.6 kbp in length, strongly cross-hybridized to the *ScADH1* coding sequences (Fig. 1A). A second fragment, about 20 kbp in length, hybridized to a lesser extent. The *Bam* HI- *Sal* I genomic fragments corresponding to 6.6 kbp were isolated from the gel to construct a DNA library pool for screening of the *ADH* gene of *P. stipitis* with *ScADH1*. Of 300 colonies screened by colony hybridization, two positive clones contained a 6.6-kbp *Bam* HI-*Sal* I fragment which hybridized to the *ScADH1* probe following Southern blot analysis. These clones were further characterized by restriction mapping and Southern hybridizations. These DNA fragments, designated *PsADH2*, strongly hybridized to the *ScADH1* probe and showed the same restriction patterns. Staggered deletions of about 500 bp each were prepared by opening the plasmid at one end of the insert, followed by timed digestions with exonuclease III (34). The deletion fragments were hybridized to the probe to localize and sequence the *PsADH2* coding regions (Fig. 2B). The coding region of *PsADH2* was tentatively located on a fragment with 4.1 and 1.5 kbp of the 5'- and 3'-flanking sequences, respectively. It was subsequently sequenced (see below).

Cloning of *PsADH1*. *PsADH1* was identified by hybridizing *Bam* HI and *Xba* I digests of *P. stipitis* genomic DNA to the coding sequences of *PsADH2*, *ScADH1*, and *ScADH2* (Fig. 1B). Two fragments, 9.4 and 6.0 kbp in length, strongly cross-hybridized to *ScADH1* and *ScADH2* (Fig. 1B, lanes 6 and 9). The hybridization of *ScADH2* to the *Xba* I genomic fragments showed that a third fragment, about 4.3 kbp in length, cross-hybridized to a lesser extent to this probe (Fig. 1B, lane 8). In addition, *PsADH2* cross-hybridized more to the 9.4-kbp band than to the 6.0-kbp band (Fig. 1B, lane 3). The putative *ADH1 gene* of *P. stipitis* hybridized more strongly to *ScADH1* and *ScADH2* than to *PsADH2*.

To isolate *PsADH1*, a DNA library was constructed as described previously and screened by colony hybridization with *SCADH2* as the probe, and positive colonies were tested further. The restriction map of this fragment differed from that of *PsADH2* in both the coding and flanking regions. The *PsADH1* gene was localized and oriented on the 6.0-kbp fragment by hybridization to *ScADH2* and sequence comparison with other *ADH* genes (Fig. 2A).

DNA sequence analysis. Analysis of the sequence of *PsADH1* revealed an open reading frame (ORF) of 1,047 nucleotides which codes for a polypeptide of 349 amino acids with a calculated molecular weight of 36,496. Potential promoter elements containing a TATA-like sequence were also found upstream from the putative ORF at position – 177 to position -183 (Fig. 3A). However, very little identity can be observed between the upstream sequences of the *ADH* genes from *P.*

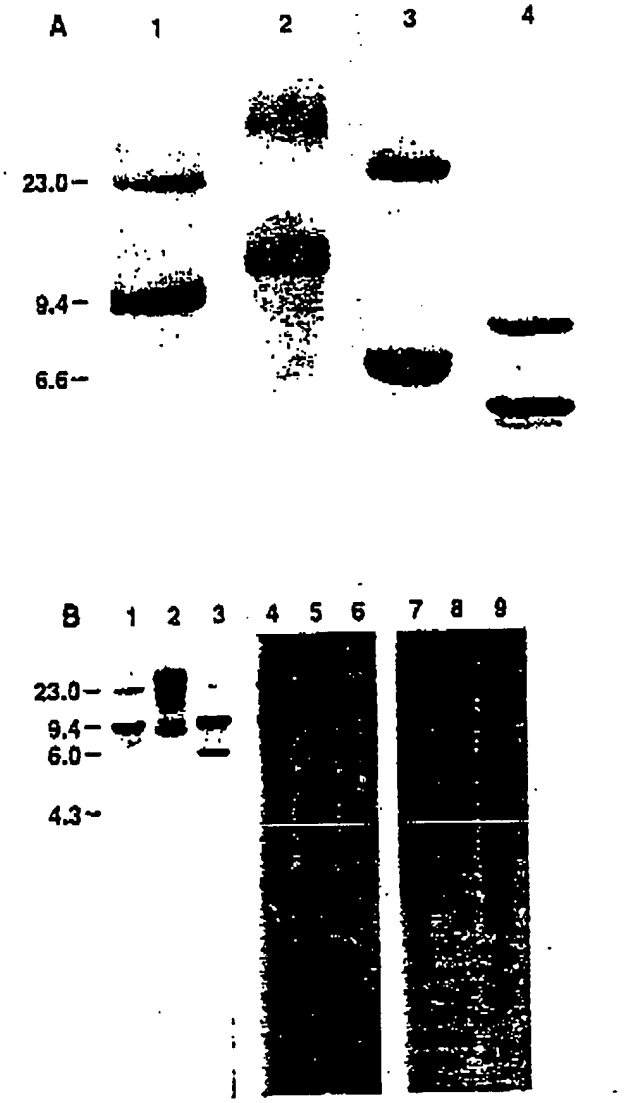

FIG. 1. Identification of PsADH DNA fragments by Southern blot analysis. *P. stipitis* CBS 6054 genomic DNA was digested with restriction enzymes indicated below and hybridized with coding sequences of the *S. cerevisiae ADH1* gene (A, lanes 1 to 4; B, lanes 4 to 6), the *S. cerevisiae ADH2* gene (B, lanes 7 to 9), and the *P. stipitis ADH2* gene (B, lanes 1 to 3). *S. cerevisiae* (A, lane 4) and *P. stipitis* genomic DNAs (20 g of each) were digested with *Bam* HI (A, lanes 1 and 4; b, lanes 1,4 and 7), *Sal* I (A, lane 2) *Bam* HI- *Sal* I (A, lane 3, *Xba* I (B, lanes 2,5 and 8), or *Bam* HI- *Xba* I (B, lanes 3, 6 and 9). the *P. stipitis ADH1 (Bam* HI- *Xba* I) and *ADH2 (Bam* HI- *Sal* I) DNA fragments were 6.0 and 6.6 kbp long, respectively. These fragments were isolated from the gel slice. The values on the left are molecular sizes in kilobase pairs.

stipitis and *S. cerevisiae*. *PsADH2* contains an ORF of 1,047 nucleotides coding for a polypeptide of 349 residues and a calculated molecular weight of 36,541. The upstream sequences of *PsADH2* show a putative TATA element (consensus sequence, TATAT/AAT/A), upstream of the RNA initiation site, at position – 110 to position – 117 (Fig. 3B).

A comparison of the polypeptides encoded by these genes with the ADH polypeptides of *S. cerevisiae* and *K lactis* showed considerable identity with the other yeast ADHs (Fig. 4). The amino acid sequences of the PsADH1 and PsADH2 isozymes are 80.5% identical to one another and are 71.9 and 74.7% identical to *S. cerevisiae* ADH1. The *P. stipits* ADH proteins are presumed to be localized in the cytoplasm, as they do not

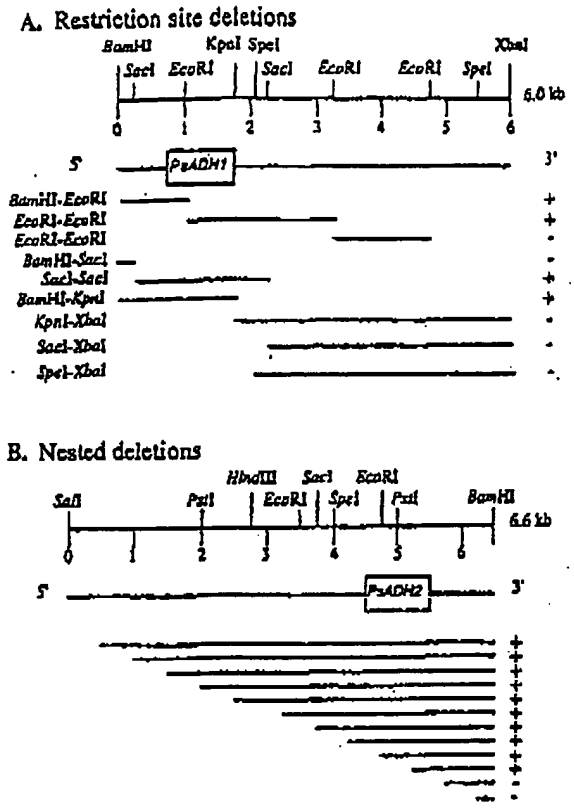

FIG. 2. Southern hybridization to identify genomic regions on clones of *PsADH1* and *PsADH2* (A) Approximate location of the *PsADH1* coding region on the 6.0-kbp *Bam* HI- *Xba* I *PsADH1* DNA fragment as determined by restriction mapping and cross-hybridisations of the resulting restriction fragments to *ScADH1*. +, positive hybridization -, no hybridization. Bold lines represent the fragment containing 5'- and 3'-flanking regions of *PsADH* genes. (B) Approximate location of the *PsADH2* coding region on the 6.6-kbp *Bam HI-Sal I PsADH2* DNA fragment as determined by nested deletions from the 5' end and cross-hybridizations of the resulting fragments to *ScADH1*. +, positive hybridization; -, no hybridization. The boxes indicate the *PADH1* (A) and *PsADH2* (B) coding regions.

possess the amino-terminal extension of mitochondrion-targeted ADHs (26, 44, 48).

Disruption of *PsADH* genes and analysis of disruptant strains. To examine the physiological role of the two *P. stipitis* ADH isozymes known, we created *Psadh* disruptant strains of *P. stipitis* by the one-step gene replacement technique (29). *P. stipitis ura3* auxotroph PSU1 was transformed with the linear construct carrying the disrupted copy of *Psadh1*, and transformants were selected on glucose medium lacking uracil. The Ura$^+$ transformants were picked randomly and screened by Southern blot analysis (Fig. 5). Genomic DNAs derived from these transformants, parental strain PSU1, and wild-type strain CBS 6054 were digested with *Sac* I. Southern blotting of CBS 6054 and PSU1 genomic DNAs with a *PsADH2* probe revealed 2.0- and 3.0-kbp bands for the *PsADH1* and *PsADH2* fragments, respectively (Fig. 5, lanes 1 and 2). Southern blotting of a Ura$^+$ transformant probed with *PsADH2* coding sequences sealed the pattern of fragments expected for a simple gene replacement at *PsADH1* with the *Psadh1::PsURA3* fragment 3.2- and 3.0-kbp bands were detected for the *Sac* I digests (Fig. 5, lane 4). The resulting *Psadh1::PsURA3/Psadh1::PsURA3* homozygous disruptant was named PSU-218, and its fermentative behavior was tested further.

In the case of the *PsADH2* disruption. *PsLEU2* was inserted into the *PsADH2* coding region (see Materials and Methods). A 4.5-kbp fragment of pJY202 containing the disrupted copy of *Psadh2* was generated by digestion with restriction enzymes *Bam* HI and *Sac* I and then transformed into the *P. stipitis ura3* Δ *leu2* auxotroph PLU20 in the presence of restriction enzymes *Bam* HI and *Sac* I. The resulting transformants were selected for growth in the absence of leucine and screened by PCR by using primers specific for the point at *which* the *PsLEU2* DNA fragment was inserted into the *PsADH2* gene. One of the transformants selected for growth in the absence of leucine, named PLU-123, showed a 2.1-kbp DNA fragment amplified by PCR, as expected for correct integration (data not shown). Correct homozygous integration of the fragment carrying the disrupted copy of *Psadh2 (Psadh2::PsLEU2)* into the corresponding genomic locus showed that 3.0-kbp fragments were replaced in the Southern blot analysis by new fragments of increased length because of the insertion of the 2.1-kbp fragments of *PsLEU2* (Fig. 5, lane 5).

We then constructed a strain lacking both *PsADH* genes. PLU-123, a derivative of PLU20 carrying disrupted copies of *Psadh2*, was transformed with the linear fragments which we previously had used to disrupt *PsADH1* in PSU1 and screened by PCR using primers specific for the point at which the PsURA3 DNA fragment was inserted into the *Psadh1 gene*. Two of the initial Ura$^+$ transformants were found to be *Psadh1::PsURA3(Psadh1::PsURA3* and *Psadh2::PsLEU2/Psadh2::PsLEU2* homozygous disruptants. The correct integration of each fragment into the corresponding genomic locus was confirmed by Southern blot analysis: 3.2-kbp *(Psadh1::PsURA3)* and 5.1-kbp *(Psadh2::PsLEU2)* fragments were detected for both *PsADH2* and *PsADH2* gene replacement (Fig. 5, lane 6). This *Psadh* multiple disruptant strain, PLU-1209, was tested along with the previously obtained disruptants for their fermentative and growth properties.

Fermentation of xylose. The *Psadh* disruptant strains were compared to appropriate wild-type and parental strains for ethanol production from xylose under oxygen limitation. Compared with those of the control strains, the apparent ethanol production and growth rate of PSU-218 were very low (Fig. 6). At the same time, the level of xylitol production of PSU-218 and double disruptant strain PLU-1209 was much higher than that of the corresponding control strains. On the other hand, disruption of *PsADH2* had little effect on growth rate, ethanol production, or xylitol production. Interestingly, *Psadh* double disruptant strain PLU-1209 failed to grow on xylose but still produced small amounts of ethanol under these conditions.

We then analyzed the ability of the *Psadh* disruptant strains to use ethanol as a sole carbon source under fully aerobic conditions. As can be seen in Fig. 7A, PSU-218 grew as well as CBS 6054 and better than its parent, PSU1, when cultivated in liquid minimal medium (YNB) containing ethanol, while PLU-1209 failed to grow under these conditions. In contrast, PLU-123 grew on ethanol at a rate similar to that of its parental strain, PLU20, indicating that PsADH2 is dispensable for growth of *P. stipitis* on ethanol unless *PsADH1* is disrupted. PLU-123, a *Psadh2* disruptant, and PLU-1209, a *Psadh1* and *Psadh2* double disruptant, were able to grow on glycerol at rates similar to those of their parental and wild-type strains (Fig. 7B). Growth of the double disruptant on glycerol indicated that its lack of growth on ethanol was not attributable to a loss of respiratory capacity.

Finally, each of the two ADH activities is able to convert ethanol to acetaldehyde and is sufficient to allow cell growth.

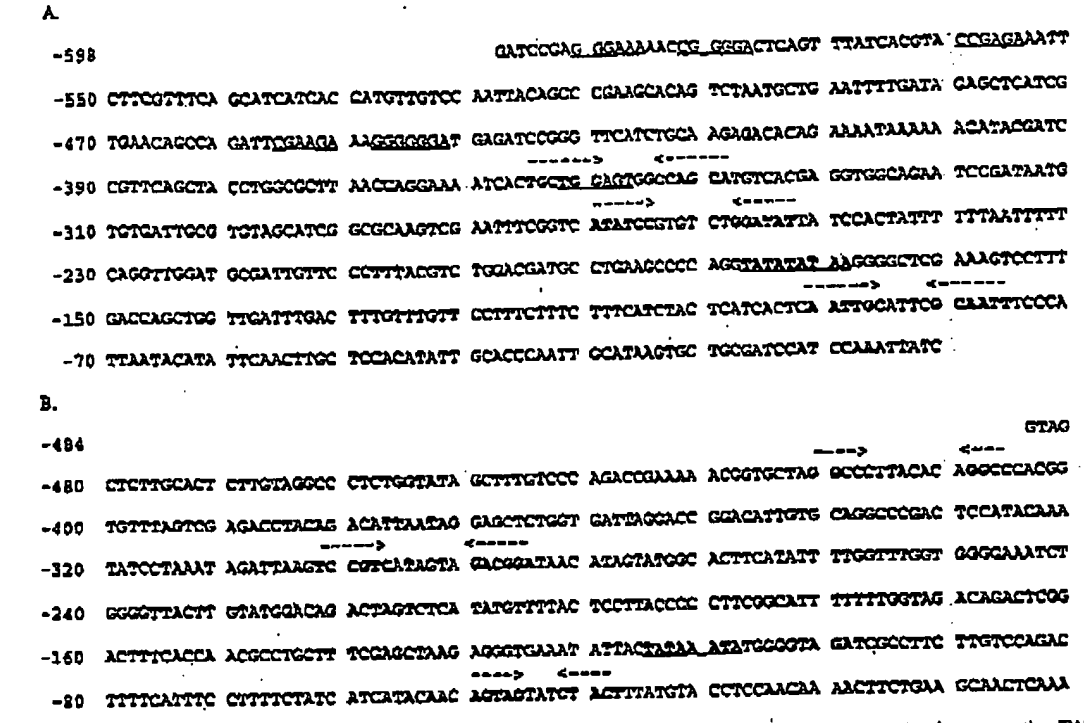

FIG. 3. Upstream sequences of *PsADH* genes and localization of their coding regions. (A) *PsADH1* upstream region showing a putative TATA element (TATATATAA, bold and underlined), hairpins (bold), and repeats (underlined). (B) *PsADH2* upstream region showing a putative TATA element (TATAAATA, bold and underlined) and hairpins (bold).

Moreover, each *Psadh* disruptant strain was still able to accumulate ethanol, although in different *amounts* (PLU-123 > PSU-218 > PLU-1209) when grown on xylose under oxygen-limited conditions.

DISCUSSION

The ADH isozymes in *P. stipitis* are encoded by multiple genes. The exact number is unknown. Hybridization of *ScADH2* to the *Xba* I genomic fragments indicated that the *P. stipitis* genome may contain three *ADH* genes. Two fragments, about 9.0 and 23.0 kb in length, strongly cross-hybridized to *ScADH2* (Fig. 1B, lane 8). A third fragment, 4.3 kbp in length, hybridized to a lesser extent. The inability of the *Psadh* double disruptant to grow on ethanol under full aerobiosis implies that no other ADH activities are present under these conditions. The gene for a third isoenzyme may be present but not expressed on ethanol under aerobic conditions.

We cloned and sequenced two structural genes from *P. stipitis*, designated *PsADH1* and *PsADH2*. They both have uninterrupted ORFs of 1,047 nucleotides and show high conservation with respect to other yeast group I ADH proteins. The two ADH isozymes of *P. stipitis* are 80.5% identical at the protein level and 79.5% identical in the coding region at the nucleotide level. *PsADH2* more strongly hybridued to *ScADH1* and *ScADH2* than to *PsADH1* (Fig. 1B, lanes 3,6, and 9). This may bean artifact of DNA conformation on that fragment, since *P. stipitis ADH* genes are structurally closer to one another than to any of the *S. cerevisiae* or *K. lactis* genes. Our designation of the isozymes as PsADH1 and PsADH2 is in keeping with the similarity of their physiological roles to those of ADH1 and ADH2 of *S. cerevisiae*. However, they appear to have evolved from one another independently of the divergence between *ScADH1* and *SCADH2* (Fig. 4). Notably, the deduced amino acid sequences of the *P. stipitis* isozymes showed a conserved amino acid residue (Lys^{295}) that is thought to be involved in the substrate-binding cleft. *S. cerevisiae* ADH1 has a methionine in this position, which is thought to contribute to the narrow substrate specificity of this enzyme (38).

Analysis of the 5'-flanking region of *PsADH1* and *PsADH2* revealed consensus sequences for a putative TATA box and an ATG context, which is optimal for highly expressed genes from *S. cerevisiae* (17a). Several direct repeats, including six direct repeats with a slight variant of the consensus sequence A/GGAGA and inverted repeats were found in the 5'-flanking region of *PsADH1*. The functional significance of these sequences has not been determined, yet it is tempting to speculate that they represent a recognition site(s) for a *trans*- acting factor(s) involved in the regulation of *PsADH1* expression Dyad symmetry is typical of the sequences recognized by dimeric DNA-binding proteins containing bacterial helix-turn-helix motifs, and the promoter region implicated in transcriptional regulation of *S. cerevisiae ADH2* also contains seven repeats with the consensus sequence A/TGGAGA and a 22-bp sequence with perfect dyad symmetry (2, 39, 49).

Bennetzen and Hall (3) suggested that there is a correlation between the expression level of a gene and its codon usage bias. The codon usage rules derived from *S. cerevisiae* genes seem to apply to *P. stipitis* as well. The *PsADH1* and *PsADH2* genes show a highly biased codon usage very similar to that observed in the corresponding genes of *S. cerevisiae* (data not shown), where only 25 preferred codons are used extensive), whereas the *URA3* gene of *P. stipitis* (47) shows a poorly biased codon usage pattern, as does the same gene of *S. cerevisiae* and *K. lactis*, where it is less highly expressed (40).

Disruption of *PsADH1* and *PsADH2* was more difficult than

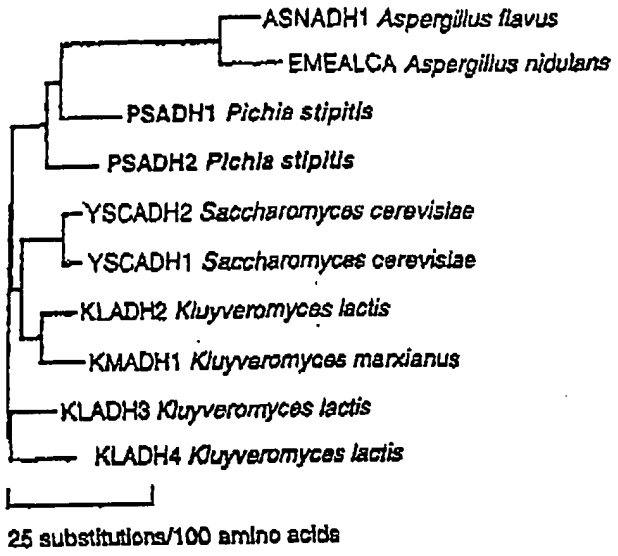

FIG. 4. Phylogenetic tree showing similarity among yeast *ADH* genes. Sequences of 10 different *ADH* genes from the GenBank and EMBL data banks were compared by using the Genetics Computer Group (12) PileUp, Distances, and Grow Tree programs. Source names are shown.

expected. Melake et al. (24) proposed that wild-type strains of *P. stipitis* are haploids. Therefore, disruption should have been easy to accomplish. However, disruption studies occasionally showed what appeared to be heterozygotes, indicating that at least some of the parental strains (or resulting disruptants) are dipioids. In contrast to that in *S. cerevisiae*, the frequeney of homologous recombination at any given locus in *P. stipitis* seems to be low and vanes somewhat from locus to locus within the genome (6). When these disruption studies were carried out, nonhomologous integration of introduced DNA was more common than homologous integration. The basis for the variability and low frequency is not clear. However, several aspects of DNA metabolism, such as DNA repair and transcription, are closely interrelated with homologous recombination (17, 37).

In the case of the *PsADH1* gene disruption, only 1 of 17 Ura$^+$ transformants was a homozygous integrant at the corresponding genomic locus. In addition to this homozygous integration, numerous *URA3* gene conversion, heterozygous, and random-integration events were detected (data not shown). Schiestl and Petes (36) found that introducing the restriction enzyme *Bam* HI together with a *Bam* HI-cut DNA fragment increased the number of transformed cells and showed that the DNA fragment was often integrated into *Bam* HI sites of the host genome. This surprising result led us to explore restriction enzyme-mediated integration in *P. stipitis* for disruption of *PsADH2*. This approach proved successful.

We created *P. stipitis* strains disrupted in each or both structural *ADH* genes. Disruption of *PsADH1* clearly affected the growth rate and ethanol production on xylose under oxygen-limiting conditions. The increase in xylitol production by the *Psadh1* disruptant indicates that in the absence of PsADH1, NADH accumulates and favors xylitol production. *PsADH1* appears to be the principal gene responsible for ethanol production because its loss results in slower growth, lower ethanol production, and much greater xylitol production under oxygen-limited conditions, whereas the loss of *PsADH2* has none of these effects. Disruption of *PsADH1* in *P. stipitis* causes this yeast to make even more xylitol than does *Candida shehaate* or *Pachysolen tannophilus*, where significant xylitol production has been observed under oxygen-limited conditions (15). Xylitol production has been explained by Debus et al. in terms of an electron sink for NADPH, generated in the phosphogluconate pathway (10). On the basis of our present studies, we propose that in *P. stipitis*, xylitol production is normally low because PsADH1 suppresses the intracellular level of NADH. When *PsADH1* is disrupted, NADH accumulates. Accumulation of NADH would, in turn, shift the equilibrium of the XDH-mediated reaction to favor xylitol over xylulose formation. Fermentative ADH activity is therefore essential not only for ethanol production but also for maintenance of redox balance in the cytoplasm. The accumulation of reducing equivalents in the form of NADH does not seem to be relieved by reoxidation under oxygen-limited conditions or by NADH-linked XR activity. The dependence of xylose metabolism on

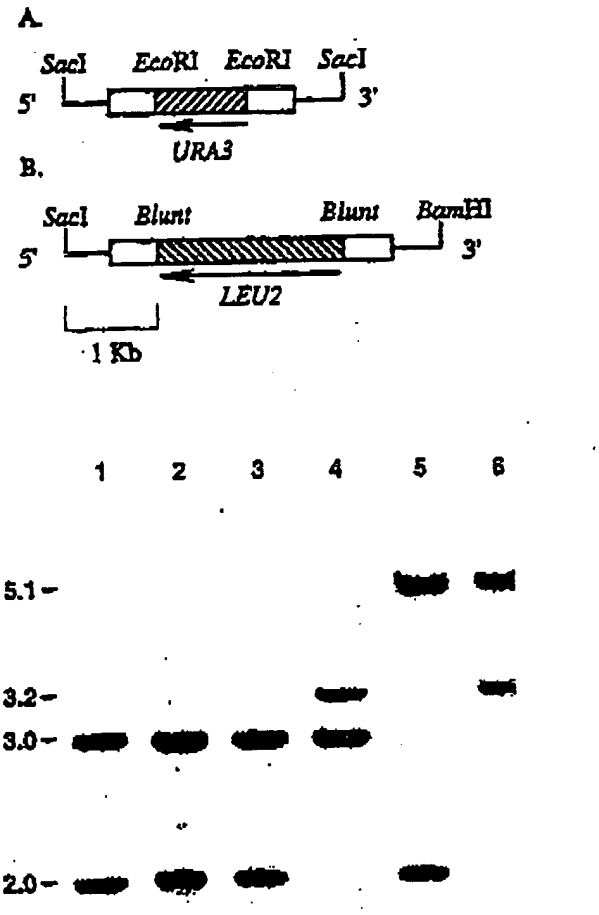

FIG. 5. Construction and Southern blot analysis confirming replacement of *PsADH* genes. Linear fragments carrying disrupted copies of the *PsADH1* (pJY102) (A) and *PsADH2* (pJY202) (B) genes were constructed and introduced into the genome as described in the text. These disruption constructs contain approximately 1.0 kbp of 5'- and 3'-flanking *PsADH1* (A) and *PsADH2* (B) homology. Bold lines represent fragments containing 5'- and 3'-flanking regions used for gene replacement. White boxes indicate *PsADH1* (A) and *PsADH2* (B) coding regions, and striped boxes represent functional *LEU2* (A) and *URA3* (B) genes from *P. stipitis*. Southern hybridization results are shown at the bottom. Strain CBS 6054 (lane 1) is the *P. stipitis* wild type. *P. stipitis* PSU1 (lane 2) and PLU20 (lane 3) are parental strains used for *PsADH1* and *PsADH2* gene replacements, respectively. PSU218 (lane 4) and PLU123 (lane 5) are strains disrupted in *PsADH1* and *PsADH2*, respectively. PLU1209 (lane 6) is disrupted in both *PsADH1* and *PsADH2*.

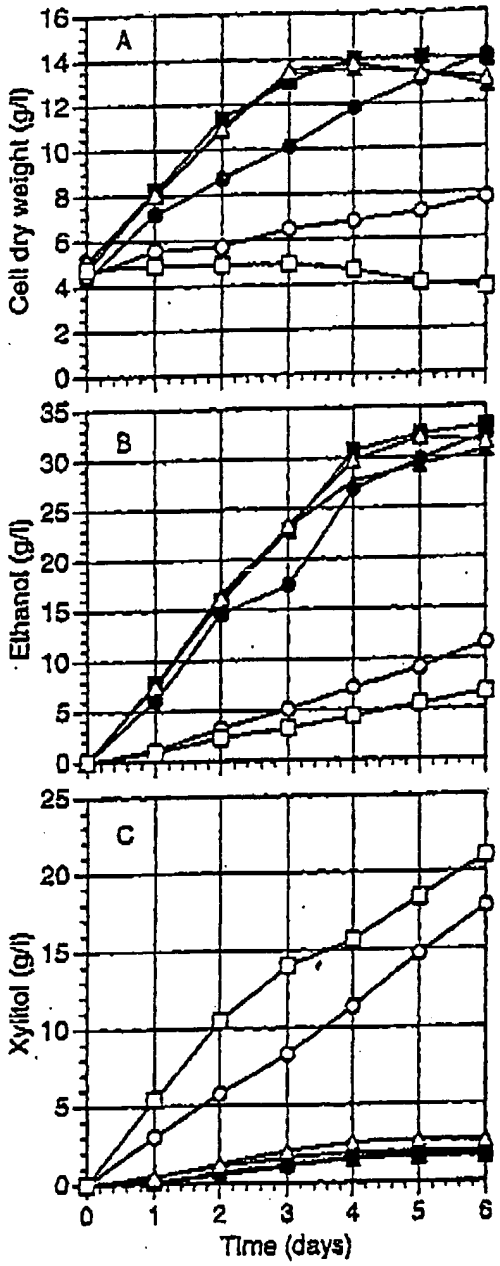

FIG. 6. Cell growth (A), ethanol production (B), and xylitol production (C) of wild-type, parental, and disruptant strains of *P. stipitis* under fermentative conditions. Inocula were grown in flasks for 48 h, and initial cell densities of each strain were adjusted to be similar. Each value represents the mean of two determinations. Symbols: ■, CBS 6054 (wild type); ●, PSU1; ▲, PLU20; △, PLU123 (*Psadh2* disruptant); ○, PSU218 (*Psadh1* disruptant); □, PLU1209 (*Psadh* double disruptant).

NADH recycling by PsADH1 could be why relatively slow growth was observed with *Psadh1* strains of *P. stipitis* (PSU-218 and PLU-1209) under oxygen-limited conditions. *Psadh1* strains still take up xylose, grow, and accumulate ethanol—albeit to a lesser extent than the parent under oxygen-limited conditions. On the other hand, the *Psadh* double-disruptant strain failed to grow on xylose and produced even less ethanol and more xylitol under these conditions. These results suggest that the PsADH2 isozyme is also involved in xylose fermentation and growth, although it probably plays a minor role compared to PsADH1.

The analysis of the *Psadh* disruptant strains, which contained either one or the other of the residual ADH activities, showed that all were able to grow aerobically on media containing ethanol as a sole carbon source. This result suggests that either PsADH1 or PsADH2 can confer the ability to oxidize ethanol. PSU-218 *(Psadh1::PsURA3)* actually grew better on ethanol than did the parental or wild-type strain, implying that *PsADH2* might be overexpressed and more important under these conditions. Because disruption of *PsADH2* had no discernible effect on fermentation or growth on xylose, an open question is whether *PsADH2* is expressed only when *PsADH1* is disrupted or whether it plays some other role.

The simultaneous presence of ethanol-producing and ethanol-oxidizing activities in the cytoplasm could result in a futile cycle. This could be why KlADH4 is compartmentalized within mitochondria in *K. lactis* (23) and catabolite repression of *ADH2* occurs in *S. cerevisiae* (30). Oxygen-dependent regulation of *PsADH* expression could avoid futile cycling in the cytoplasm of *P. stipitis* if respirative enzyme activities were repressed and fermentative enzyme activities were induced under oxygen-limited conditions. In *P. stipitis*, a Crabtree-neg-

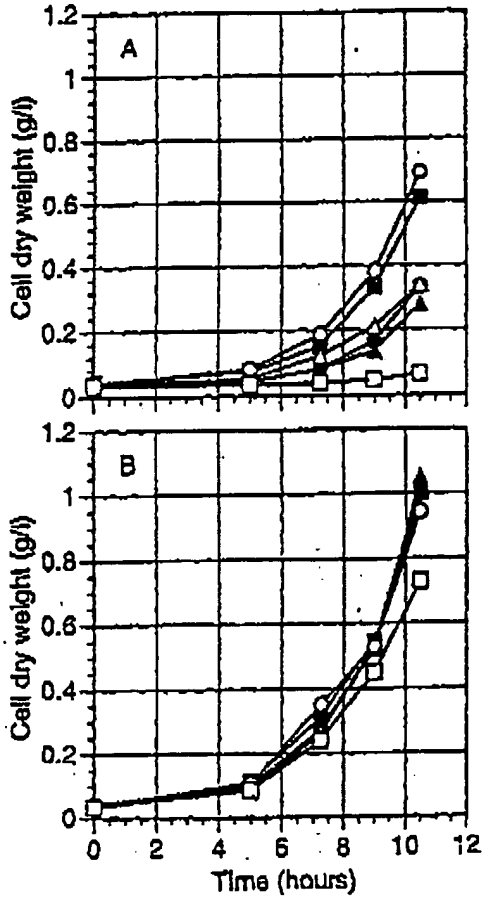

FIG. 7. Aerobic growth of *P. stipitis* wild-type CBS 6054, parental, and *Psadh* disruptant strains on either ethanol (A) or glycerol (B). Cells were grown in YNB with either 2% ethanol or 3% glycerol plus 100 μg of leucine or uridine/ml, as needed, with shaking in a 125-ml baffled flask at 180 rpm and 30°C. Symbols: ■, CBS 6054 (wild type); ●, PSU1 (*ura3-2/ura3-2*); ▲, PLU20 (*ura3-3/ura3-3 Pleu2Δ-1/Pleu2Δ-1*); △, PLU123 (*Psadh2* disruptant); ○, PSU218 (*Psadh1* disruptant); □, PLU1209 (*Psadh* double disruptant).

ative yeast, fermentation enzymes such as ADH and PDC are induced only under oxygen limitation (25), and no fermentation occurs on medium containing 3% xylose under fully aerobic conditions (7). This is in keeping with a hypothesis of oxyen-dependent regulation of *ADH* expression in *P. stipitis*, as discussed above. The different behavior of these yeasts compared to that of *S. cerevisiae* is most probably explained by differences in the nature of the fundamental mechanisms by which fermentation is regulated.

The *Psadh* double-disruptant strains are not able to utilize ethanol, indicating that no other important ADH activities are present in *P. stipitis* when it is growing on ethanol under aerobic conditions. However, the *Psadh* double disruptants still produce ethanol from xylose, implying that a third enzyme might be expressed under oxygen-limited conditions. ADH null strains of both *S. cerevisiae* (14) and *K. lactis* (31) can still produce residual amounts of ethanol when grown aerobically on 2% glucose. The biochemical basis of this phenomenon remains unclear. In any case, the addition of a respiratory inhibitor such as 2,4-dinitrophenol, sodium azide, or potassium cyanide should immediately block the production of ethanol in the *Psadh* null strain because unimpaired mitochondrial function is necessary for growth on and alcoholic fermentation of xylose in *P. stipitis* (20). The function of this respiratory activity is not clear, but it could be necessary to generate energy for xylose transport (19). Experiments are under way to unravel the molecular basis for the expression of *PsADH* genes in this least.

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